IN SITU HYBRIDIZATION OF 70 kD HEAT SHOCK PROTEIN mRNA IN A RAT MODEL OF ETHANOL SELF-ADMINISTRATION

THESIS

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Sucrose fading was used to initiate self-administration of ethanol on an FR4 schedule in male Fischer 344 rats. Rats showed low response rates for ethanol alone. After administration of liquid diet containing ethanol, ethanol intake increased over levels prior to administration of the liquid diet.

In situ hybridization compared mRNA for the inducible or constitutive 70 kD heat shock proteins in ethanol and nonethanol rats. Both inducible and constitutive mRNAs were found in nonethanol and ethanol tissues, possibly due to physiological changes resulting from the liquid diet or from the stress of handling the animals. In peripheral organs, radiolabeling was higher in ethanol tissues. In brain regions, nonethanol tissues showed higher radiolabeling.

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LIST OF ABBREVIATIONS

A	adenine
AMP	adenosine monophosphate
ATP	adenosine triphospate
BiP	binding protein
C	cytosine
°C	Celsius
cDNA	complementary DNA
Ci	Currie
dATP	deoxyadenosine triphosphate
DEPC	diethylpyrocarbonate
dL	deciliter
DNA	deoxyribonucleic acid
DTT	dithiothreitol
FR	fixed ratio
G	guanine
g	gram
GRP	glucose regulated protein
HSC70	70 kD constitutive heat shock protein
HSE	heat shock element

HSF	heat shock factor
HSP	generic term for heat shock protein
HSP10	10 kD heat shock protein
HSP60	60 kD heat shock protein
HSP70	70 kD inducible heat shock protein
kb	kilobase
kD	kilodalton
kg	kilogram
M	molar
mg	milligram
mL	milliliter
µL	microliter
mmol	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
PBS	phosphate buffered saline
pg	picogram
pmol	picomole
rRNA	ribosomal ribonucleic acid
SSC	standard sodium citrate
T	thymine

v/v	ratio of volume to volume
w/v	ratio of weight to volume

CHAPTER 1

INTRODUCTION

Chronic ethanol abuse produces biochemical adaptations in the central nervous system which underlie the development of tolerance to and dependence on alcohol. Alcoholics consequently suffer withdrawal symptoms when deprived of ethanol. The mechanisms underlying the effects of ethanol on the central nervous system are poorly understood.

Ethanol has also been shown to cause changes in abundance or functioning of various receptors, neurotransmitters, ion channels, and membrane transport systems (Parent et al., 1987). Recently several investigators have reported ethanol-induced changes in specific mRNA or protein abundance; among these proteins are the stress proteins, also commonly known as heat shock proteins or HSPs (Miles et al., 1991; 1992).

This research was conducted to answer three questions. Could stable self-administration of ethanol be maintained in the Fischer 344 strain of rats? Were self-administered ethanol concentrations altered after chronic exposure to ethanol? Were levels of stress proteins affected by chronic exposure to ethanol? To answer these questions, the following experiment was performed. Male rats of the Fisher 344 strain were trained to self-administer ethanol via a sucrose fading technique (Samson, 1986) in daily 30 minute training sessions.

After stable responding was maintained and a dose effect curve generated, the animals were chronically exposed to ethanol via a liquid diet containing 5% (v/v) ethanol (Lal et al., 1988). Another dose effect curve was established to ascertain the effects of long term exposure to ethanol. The animals were allowed several weeks without ethanol; then they were again fed liquid ethanol diet for two weeks, while rats which had never been exposed to ethanol were fed liquid diet without ethanol. *In situ* hybridization was used to assess the effects of ethanol on levels of mRNA for the 70 kD heat shock proteins, using radiolabeled probes specific for either the inducible or constitutive form (commonly referred to in literature as HSP70 and HSC70, respectively), in tissue sections from brain, liver, kidney, gastrointestinal, heart and skeletal muscle. Intensity of radiolabeling was compared in animals fed liquid diet with or without ethanol.

Self-Administration of Ethanol

Animal models of chronic ethanol exposure have been used as a pharmacological correlate to the study of alcoholism in humans. Maintenance of animal behavior with ethanol reinforcement has been demonstrated by a variety of procedures: intravenous route of self-administration, intragastric route of self-administration, and the oral route of self-administration. Little success has been achieved with the oral route unless food and/or fluid deprivation are used both to initiate and maintain ethanol self-administration. Thus, the

reinforcing properties could be related to ethanol's calories rather than any direct pharmacological effects (Samson, 1986). Oral self-administration in animals that have been neither food nor fluid deprived suggests that caloric need is not required to initiate and maintain oral ethanol reinforcement in the rat. However, ethanol's caloric content cannot be ruled out as a potential component of the mechanism of ethanol reinforcement.

Samson (1986) proposed a method of sucrose fading to initiate and maintain stable responding for ethanol. Rats were taught to lever press for presentation of a dipper containing a 20% (w/v) sucrose solution. Over a period of time, ethanol concentration was increased while sucrose decreased until only ethanol was presented as the reinforcing stimulus. This gradual replacement eliminated the problems inherent with training an animal with a sucrose/ethanol solution then suddenly testing with only ethanol, i.e., the rapid taste change may have inhibited the rat's responding for ethanol. Different strains of rats have shown differing preferences for alcohol and a strain of ethanol-preferring rats has been established (Froehlich et al., 1988). Rats of the Fischer 344 strain have shown less responding for ethanol as compared to other rodent strains (George, 1987; Suzuki et al., 1988), with ethanol serving as a weak reinforcer. Sucrose fading could possibly overcome this strain's initial aversion to the taste or smell of ethanol.

Traditionally in animal behavioral models, tolerance to a drug has been demonstrated by a shift to the right in the pretolerant dose effect curve such

that a higher concentration of drug produced the same pretolerant effect or response when response is plotted against concentration. Conversely, sensitization is demonstrated by a shift to the left of the pretolerant dose effect curve. Thus, to assess the behavioral changes in our subjects' responding for ethanol, a pre-diet (pre-chronic) dose effect curve was established to document response at various ethanol concentrations. The animals were chronically exposed to ethanol via an ethanol diet (Lal et al., 1986) and a post-diet level of response and dose effect curve were established.

Lal et al., (1988) administered an ethanol liquid diet chronically for 6 days, during which time rats consumed an average ethanol dose of 2.5 g/kg/day. Blood ethanol concentration fluctuated in a daily pattern from 0.8 - 2.0 mg/mL (80 - 200 mg/dL). The minimal value was obtained when food was introduced, the maximal value 12 hours later. Minimum values increased over 1 mg/mL by the end of the fifth day. Rats fed diet for 3 days experienced withdrawal as measured by anxiety. Tolerance occurred at 5 days to the intoxicating effects of ethanol.

Majchrowicz (1975) concluded that continuously sustained elevation of blood ethanol concentration for 4 days is sufficient for the induction of physical dependence upon ethanol and for manifestation of fully developed withdrawal signs and symptoms. Dependence was induced by intragastric intubation of 20% (w/v) ethanol solutions at 9 - 15 g/kg in 3 - 5 fractional doses daily for 4 days. Onset of ethanol dependence was marked by the progressive

appearance of signs and reactions of an ethanol withdrawal syndrome even before complete clearance of ethanol from the blood, when blood ethanol concentration was 100 mg/dL. Tail tremors and tail stiffness, general tremors and spasticity, wet shakes and chattering teeth, induced convulsions, and running episodes marked the withdrawal syndrome. General hyperactivity occurred 0 - 6 hours after clearance of ethanol from the blood. Blood ethanol concentration of approximately 200 mg/dL was an intermediate neutrality stage characterized by definitive recession of the signs of overt intoxication and a subtle onset of progressively severe signs and reactions of ethanol withdrawal syndrome. The definitive reversal in central nervous system functions from the extremes of ethanol intoxication to ethanol withdrawal occurred within a relatively short period of time (1 - 2 hours). A fully developed withdrawal syndrome, including convulsive seizures, occurred at blood ethanol concentration as high as
100 mg/dL.

To relate these figures to humans, patients tested after alcohol-related motor vehicle accidents showed median alcohol concentrations at 200 mg/dL. Concentrations of 50 mg/dL are significantly associated with an increased risk of injury-causing automobile accidents. The range of concentrations in patients tested was from 0 - 550 mg/dL. For a 73 kg man, 340 g of 80 proof liquor in 1 hour on an empty stomach is sufficient to achieve blood alcohol level above 200 mg/dL (Chang and Astrachan, 1988).

In this investigation, ethanol consumption was determined solely by the amount of ethanol solution or liquid diet ingested per day. The procedure of collecting blood by restraining the animal and nicking the tail would likely have produced anxiety, which could have affected levels of stress proteins. It has been reported that restraint anxiety alone is sufficient to induce stress proteins (Blake et al., 1991).

Changes in membrane receptors, membrane fluidity and neuro-transmitters, as well as other effects of ethanol, seemed likely to affect the levels of stress proteins. Numerous studies have linked ethanol to the induction of heat shock proteins (HSPs), but none so far has developed an *in vivo* model (Li, 1980; Miles et al., 1991, 1992; Chelbi-Alix and Chousterman, 1992; Nakamura et al., 1991; Hahn et al., 1991; Omar et al., 1990; Walsh and Crabb, 1989; Zatloukal et al., 1988; Rodenheiser et al., 1986; Koskinas et al., 1993).

Stress Proteins

Living organisms respond at the cellular level to unfavorable conditions such as heat shock or other stressful situations of many different origins by the rapid, vigorous, and transient acceleration in the rate of expression of a small number of specific genes, which leads to the appearance of stress proteins (Morimoto et al., 1990). This classic stress response can be induced by many factors in addition to heat; thus, the terms "heat shock protein" and "stress protein" are often used interchangeably in the literature. The stress response

shows many similarities regardless of the inducer. Because the levels of HSPs are increased during stress, it is assumed the stress proteins play a "protective" role in the cell, stabilizing cellular components against damage.

The stress response and the stress proteins themselves are highly conserved from prokaryotes to eukaryotes. The most common stress proteins belong to the 70 kD protein family (Table 1), which is distinguished by extensive

Table 1: The HSP70 family of related ATP-binding proteins present within different intracellular compartments (Morimoto et al., 1990).

HSP73:	An abundant and constitutive protein present in the cytoplasm and nucleus. Implicated in the uncoating and/or reformation of clathrin-coated vesicles and in the maintenance of a translocation-competent state of certain proteins that are transferred across intracellular membranes.
HSP72:	Negligible levels in the normal cell, but induced to high levels after stress. Present within the nucleus, nucleolus, and cytoplasm. Likely to function in the stressed cell, similarly to HSP73, to maintain the solubility of cytosolic and nuclear proteins as well as perhaps facilitate the removal of denatured proteins.
GRP 80 kD or BiP:	Abundant constitutive protein present within the lumen of the endoplasmic reticulum. Facilitates the proper assembly of monomeric proteins into larger macromolecular complexes.
GRP 75 kD:	Constitutive protein localized within the mitochondria, most likely present in the matrix. Presumed to function similarly to BiP, in the proper assembly of mitochondrial monomeric proteins into larger macromolecular complexes.

gene and functional similarity to HSP70. The highly inducible form is a 72 kD protein synthesized at very high levels after stress, commonly called HSP70. The 73 kD protein is the constitutive form, HSC70, an abundant protein of the normal, unstressed cell. However, both the inducible HSP70 and the constitutive HSC70 comprise multiple isoelectric forms falling between pH 5 and pH 7.

Proteins from the HSP70 family are single polypeptides of approximately 650 amino acids with two functional domains. The HSP70 substrate-binding domain maps to the carboxy-terminal region with residues 384 - 470 making up the segment that is similar to the α_1 and α_2 domains of the human leukocyte antigen heavy chain. The structure of a proteolytic fragment representing the amino terminal 382 residues (the ATP-binding domain) has been found to closely resemble that of actin, although this remarkable similarity in quaternary structure is not reflected in the amino acid sequence.

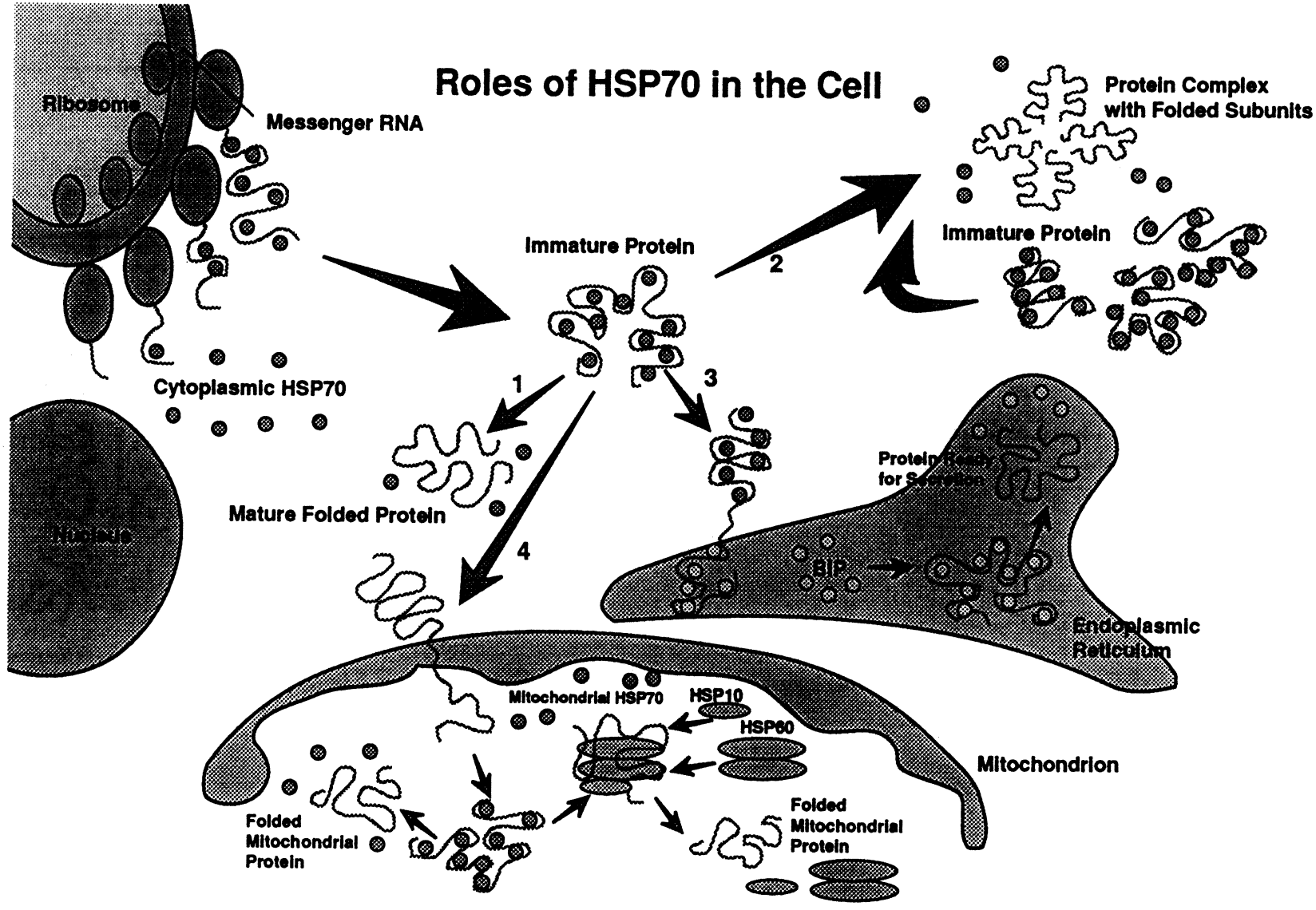
Immunological, biochemical, and DNA sequence analyses have demonstrated that HSP70 and HSC70 are highly related proteins but distinct gene products. Antibodies have been developed that do not show cross-reactivity between HSP70 and HSC70 (Craig, 1985). Both proteins exhibit numerous common properties: stoichiometric copurification during gel filtration or ion exchange chromatography, similar intracellular locales, and high affinity for various nucleotides, the highest being ATP. They are methylated polypeptides and appear to bind fatty acids. To date, no obvious biochemical

or biological differences between the constitutive HSC70 and the highly stress-inducible HSP70 have been described. HSP70 and HSC70 are involved in protein-protein interactions, assuring proper protein folding, assisting in protein transport across membranes and possibly protecting proteins during stress (Figure 1).

In vitro, HSP/HSC70 proteins facilitate the uncoating and release of clathrin coated triskelions from clathrin-coated vesicles in the presence of ATP. With the removal of the clathrin triskelions, the vesicles can fuse with other intracellular organelles, such as lysosomes. The HSPs appear to remain bound to the released triskelion.

A major subset of heat shock proteins assist other polypeptides to maintain, or assume, a conformation required for their correct assembly into biologically active structures or localization. By definition, chaperones are proteins which assist the assembly of some oligomeric proteins but are not components of the final structure. Bacterial and mitochondrial chaperonins, a group of chaperones, are induced as stress response proteins. In addition, they are expressed constitutively during normal cell growth and are essential for cell viability. The chaperonin folding activity may be important for proteins imported into organelles or released from ribosomes with an incomplete three-dimensional structure and also in anti-folding to maintain polypeptides in atranslocation competent conformation until they have entered the secretory pathway.

Figure 1: Roles of HSP/HSC70 in the cell (Welch, 1993). HSP70 is shown in the figure, but HSC70 likely performs the same functions. Several pathways for folding and distributing proteins inside cells are managed by stress proteins. The cytoplasmic form of HSP/HSC70 binds to proteins being produced by the ribosomes to prevent their premature folding. The HSP may dissociate from the protein, allowing it to fold itself into its functional shape (1) or to associate with other proteins forming larger, multimeric complexes (2). If the protein is destined for secretion, it may be carried to the endoplasmic reticulum and given to BiP or another related stress protein that directs its final folding (3). Other proteins are transferred to mitochondria or other organelles (4). Inside the mitochondrion, another specialized form of HSP70 sometimes assists the protein in its final folding, but in many cases, the protein is passed on to a complex of HSP60 and HSP10. The HSP60 molecule seems to serve as a "workbench" on which the mitochondrial protein folds.


Roles of HSP70 in the Cell
Ribosome
Messenger RNA
Cytoplasmic HSP70
Immature Protein
1
2
3
4
Protein Complex
with Folded Subunits
Immature Protein
Mature Folded Protein
Nucleus
BiP
Endoplasmic
Reticulum
Mitochondrial HSP70
HSP10
HSP60
Mitochondrion
Folded
Mitochondrial
Protein
Folded
Mitochondrial
Protein

More than 95% of mitochondrial proteins are encoded by nuclear genes, synthesized as precursor proteins on cytosolic polysomes and eventually imported into one of the four mitochondrial subcompartments (outer membrane, intermembrane space, inner membrane and matrix). Proteins are not translocated in their mature (folded) conformation across the mitochondrial membranes, but instead are in a loosely folded, or unfolded, conformation that is compatible with translocation across the membranes (Figure 1). Proteins are refolded upon import into the mitochondria. ATP and HSPs are involved in both processes. Yeast mutants deficient in HSP70 were found to be defective in mitochondrial protein import. In addition, translocation of precursor proteins into isolated mitochondria was stimulated by addition of HSP70 (Morimoto et al., 1990).

HSP70 also stimulated protein translocation into the endoplasmic reticulum *in vivo* and *in vitro*, implying a possibly similar mechanism for an initial reaction of protein translocation across various membrane systems (Figure 1). It remains to be elucidated whether HSPs and other cytosolic factors are required only for import of some precursor proteins or if they play a more general role in import of a large number of precursor proteins.

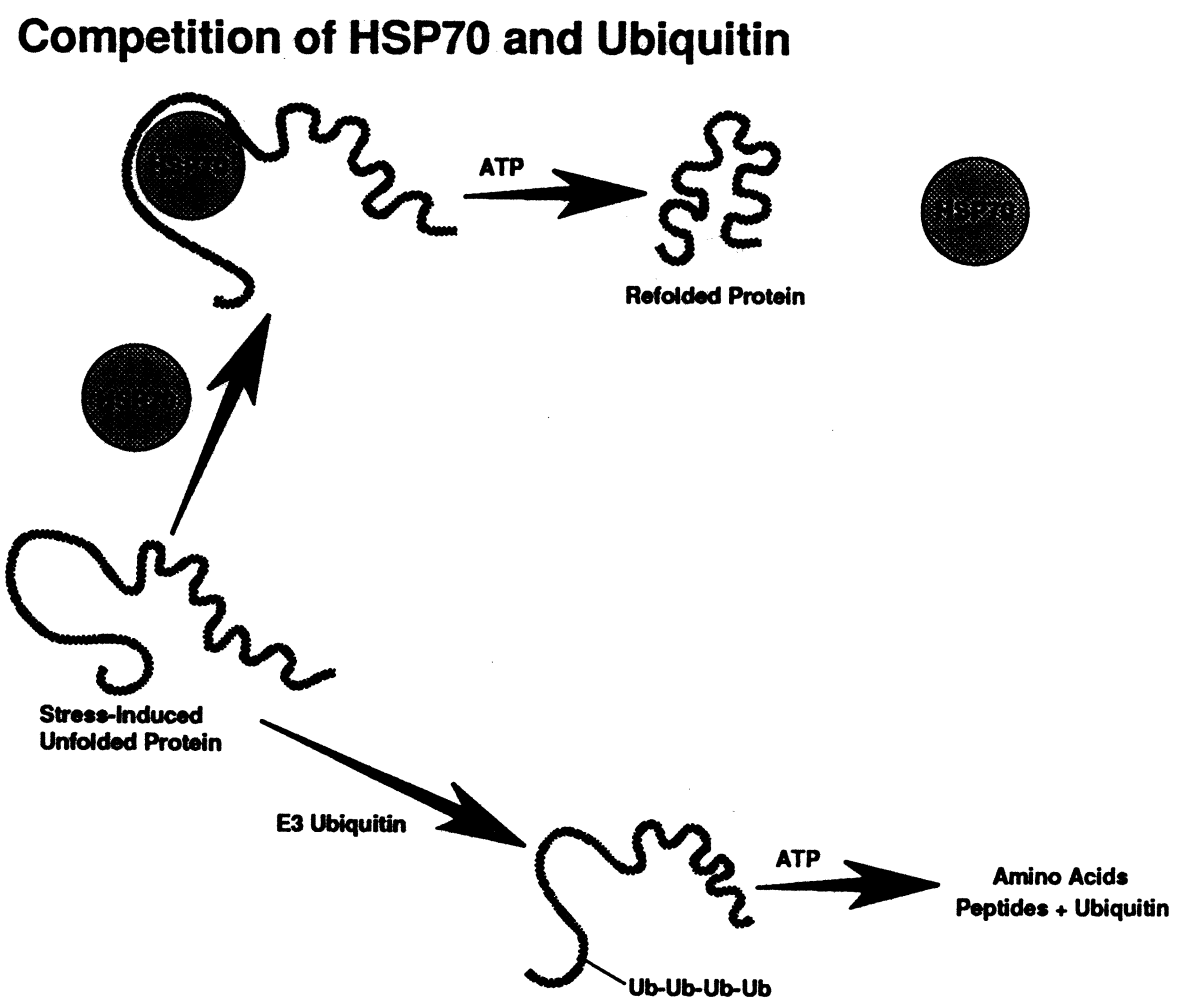
Schlesinger et al., (1990) proposed that the functions of the stress induced proteins are to protect critical cellular proteins from irreversible denaturation and thus allow them to regain a native, active configuration when stress has been removed. HSP70 functions as a chaperone to form complexes

with proteins that misfold or unfold during stress thereby rescuing these proteins from irreversible damage and degradation. Not all stress-damaged proteins can be rescued, however (Figure 2). Stressed cells also activate several components of a proteolytic ubiquitin degradation system that normally functions to turn over cytoplasmic and nuclear proteins in all eukaryotic cells. Ubiquitin could bind to unfolded or misfolded proteins and mark them for proteolysis. Formation of an HSP70-protein complex would rescue the protein whereas complex with ubiquitin would lead to protein degradation. Levels of mRNAs for ubiquitin increase almost 3-fold after stress in yeast. Both HSP70 and ubiquitin were increased in patients with amyotrophic lateral sclerosis (Garofalo et al., 1991).

Ubiquitinization is essential for DNA to be active. In DNA structure, ubiquitin was found covalently bound to histones H2A and H2B. In heat shocked cells, ubiquitinated histones disappear rapidly. This activity is likely associated with the condensation of chromatin detected in stressed mammalian cells and to the rapid decrease in DNA synthesis after stress.

Ubiquitin also covalently binds to two ribosomal proteins. Ribosome assembly is quite sensitive to stress and ribosomal RNA processing slows after shock with an accumulation of the large precursor RNAs. Shortly after stress, the major inducible heat shock protein, HSP70, moves to the nucleolus (the sight of ribosome biogenesis) where it binds tightly to proteins in that organelle. HSP70 is involved in recovery of nucleolar function, i.e., ribosomal RNA

Figure 2: Competition of HSP/HSC70 and ubiquitin for unfolded proteins in the stressed cell (Schlesinger et al. 1990). Alternate fates for an unfolded protein in a stressed cell: binding with HSP70 allows the protein to refold properly. If the unfolded protein complexes with polyubiquitin, the protein is degraded into amino acids and peptides.



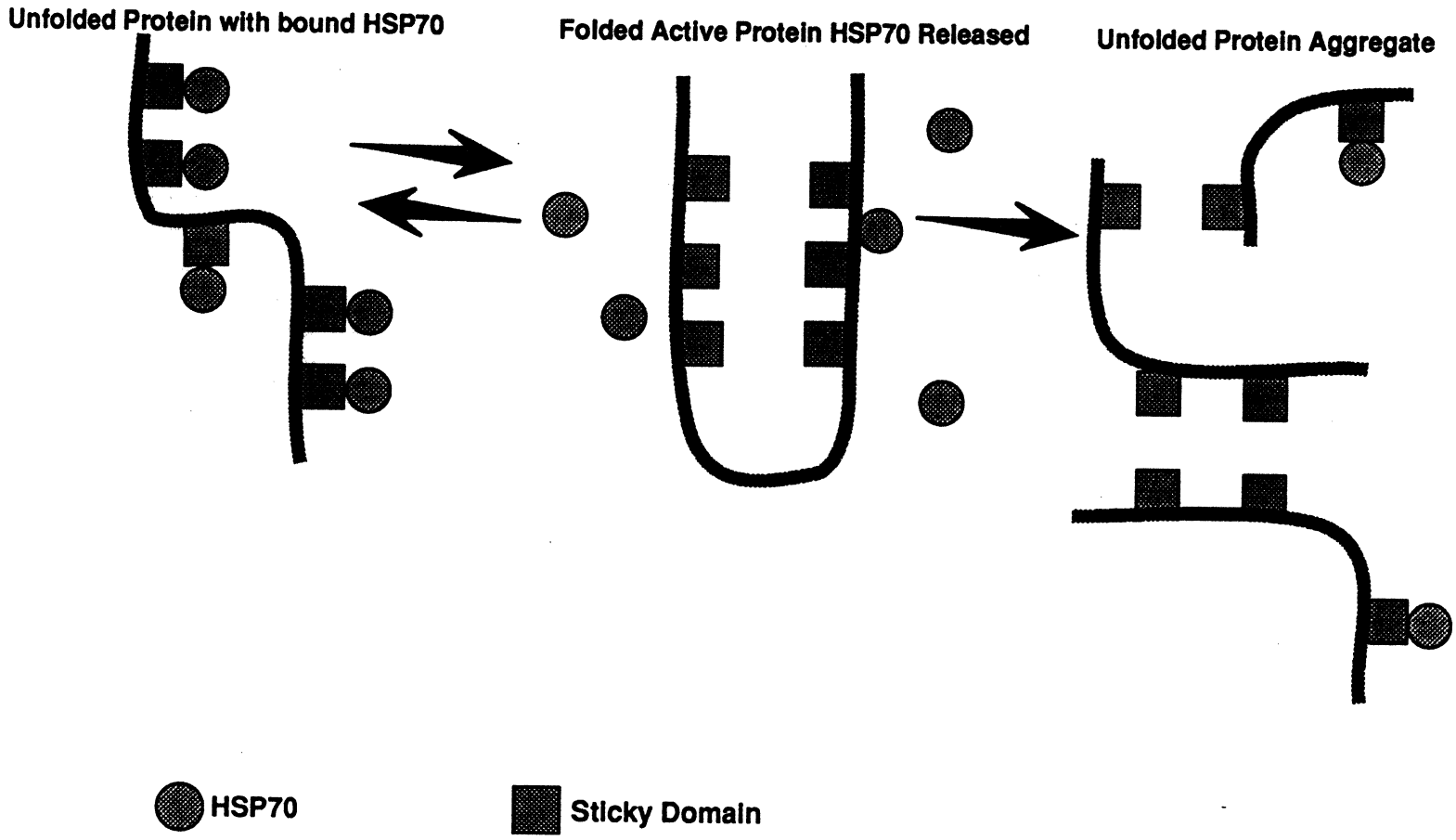
synthesis and processing. Association of HSP70 with components involved in protein synthesis argues that this protein is involved with translation following heat shock.

The continued formation of unfolded proteins in stressed cells increases the demand for ubiquitin, which is supplied by activation of a polyubiquitin gene. Several ubiquitin molecules form aggregates, which are recognized and degraded by an ATP-dependent proteasome, eliminating the ubiquitin complexes that accumulate in stressed cells (Schlesinger et al., 1990).

In an unfolded or improperly folded protein, hydrophobic or "sticky" regions would become available to bind with HSP/HSC70 or other unfolded protein regions (Figure 3). Abnormal proteins may form insoluble aggregates resulting from incorrect folding of the polypeptide chains. These aggregates could be recognized by ubiquitin. Some hydrophobic domains would bind the HSPs and others would promote aggregation. The pool of available HSP/HSC molecules would be depleted. HSP/HSCs of the 70 kD family start to aggregate *in vitro* upon heating at stress temperatures. Depletion of various "available" HSPs might be the basis for heat shock gene activation. Conversely, excess "available" HSPs could inhibit the expression of the corresponding genes and account for the observed apparent self-regulation (Schlesinger et al., 1990). Protein-protein interactions of HSPs depend on the ability to recognize unstructured "sticky" protein regions. The trigger for the stress response, the induction of HSP70, is believed to be denatured proteins or other protein

Figure 3: Protein interactions of HSP70 (Schlesinger et al., 1990). Cellular proteins are expected to show a conformational equilibrium between folded and partially unfolded forms. HSP70 binds to unfolded regions and prevents protein aggregation. This binding is reversible (after ATP hydrolysis for HSP/HSC70). Heat shock or addition of solvents such as ethanol favors the unfolded conformation and aggregation occurs at a faster rate than chaperoning (binding to unfolded regions). Increasing the pool of free HSPs by a "priming" stress favors chaperoning, which delays aggregation.

Protein Interactions of HSP70



modifications, such that "sticky" regions of denatured proteins are exposed and bind to HSP70.

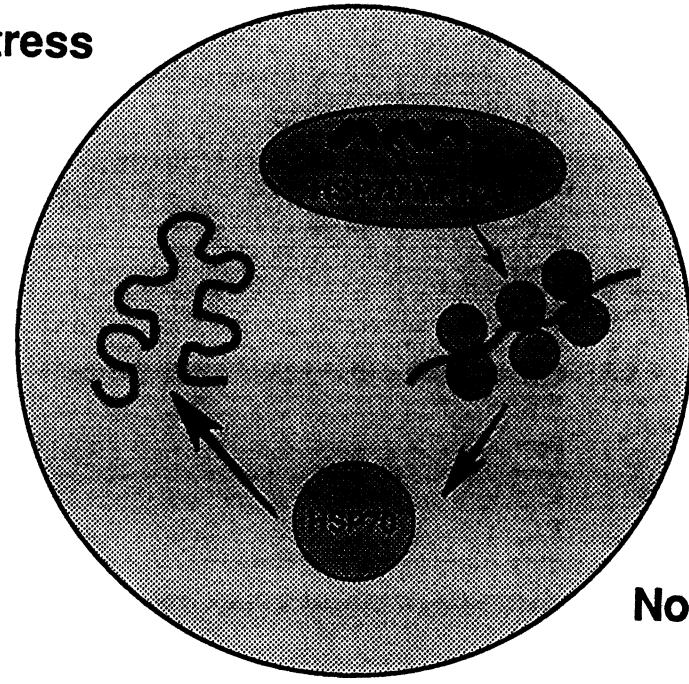
Stress proteins are induced by many factors (Figure 4). Each of these inductions could be explained by the depletion of a pool of available stress proteins needed to stabilize cellular proteins. Ethanol and other particularly longer-chain aliphatic alcohols, cocaine, arsenite, carbonyl cyanide, m-chlorophenyl hydrazone, diamide, dimethylsulfoxide, hydrogen peroxide, heavy metals (Hatamaya et al., 1992), cadmium, prostaglandins, amino acid analogs, certain ionophores, exercise, oxidative stress, and inhibitors of mitochondrial function induce HSP70. In some cases, viral infections, fever, inflammation, metabolic diseases, prolonged seizures (Sharp et al., 1992) and cell and tissue damage have been shown to induce HSP70 (Morimoto et al., 1990; Schlesinger et al., 1990). Oxidant injury, hypoxia (Benjamin et al., 1992), cardiac hypertrophy, and ischemia also lead to higher levels of HSP70. Benjamin's data indicate the effects of ATP depletion alone are sufficient to induce the DNA binding of heat shock factor (or HSF, discussed below) when oxidative metabolism is impaired, and are consistent with a model proposed recently for transcriptional regulation of stress protein genes during ischemia. Blake et al., (1991) found that restraint stress, known to activate the hypothalamic-pituitary-adrenal axis, induces expression of HSP70 mRNA selectively in the adrenal cortex of rat. Some inductions of HSPs may be mediated by stress-induced

Figure 4 : Inducers of the stress response (Schlesinger et al., 1990). Stressful conditions induce the transcription of HSP70 mRNA, which is translated into the HSP70 protein. The HSP70 protein then interacts with immature or unfolded proteins to stabilize the proper protein folding.

Inducers of Cellular Stress Response

Environmental Stress

Transition Heavy Metals
Amino Acid Analogs
Heat Shock
Pathophysiological State



Inhibitors of Energy Metabolism

Fever
Hypertrophy
Oxidant Injury
Ischemia
Viral Infection
Inflammation
Anti-Neoplastic Chemicals

Non-Stressful Conditions

Cell Cycle
Growth Factors
Oncogenes and Proto-Oncogenes
Development and Differentiation

increases in adrenocorticotropic hormones as well as direct effects of the stress agent on the cell (Blake et al., 1994).

Foreign, or nonfunctional, proteins introduced into cells by a variety of mechanisms induce HSPs. Several pieces of evidence suggest that proteins embedded in lipids are of primary concern. The concentration of aliphatic alcohols required for the induction of stress tolerance (discussed below) was related to their water-lipid partitioning coefficient. The greater the partitioning of alcohol into lipids, the lower the required concentration in the aqueous medium. Whether all of the various agents induce the response via independent pathways or converge within a single point along one pathway is unresolved (Morimoto et al., 1990).

Although heat is the most common experimental inducer of HSPs, stress proteins are not induced by heat during early embryonic stages. In rats, HSP70 is not induced in the brain until 3 weeks postpartum, whereas other organs show no difference in induction. Most homeothermic cells, following a temperature upshift, remain in a heat shock response. They continue to express high levels of the HSPs but eventually die if left at the higher temperature (Morimoto et al., 1990).

Certain inducers of the stress response appear to have an additive effect. The stress response is not induced below certain temperatures or ethanol concentrations (4% in culture cell media). However, if the low temperature is paired with the low ethanol concentration, the stress response is induced

(Rodenhiser et al., 1986). Hahn et al., (1991) found similar results with nicotine in combination with either alcohol or heat. Thermal denaturation has been proposed for the rate limiting step in hyperthermic cell killing. Ethanol in culture medium sensitizes cells to heating and favors thermal protein denaturation *in vitro* via additive effects that lower the onset temperature for denaturation in the presence of ethanol (Massicotte-Nolan et al., 1981).

Once the stress response is induced, cells show a lingering resistance to further stress (referred to as tolerance in the literature, referred to here as stress tolerance to distinguish from tolerance to the intoxicating effects of ethanol), presumably because the stress proteins are still being expressed (Nakamura, 1991). The initial or secondary inducer could be heat (thermo-tolerance), ischemia, chemical, drug, etc. Stress tolerance was first demonstrated with survival studies. Ethanol was commonly used as an initial inducer (Li and Hahn, 1978; Li, 1980). Stress tolerant cells recover their cytoskeletal morphology and normal macromolecule synthesis faster than nontolerant cells. A "priming" stress also attenuates the inhibition of protein synthesis which occurs during and after heat shock. Less inhibition of normal RNA processing is observed in cells first made thermotolerant and then exposed to a subsequent heat shock challenge.

The interplay between HSPs and stress induced protein unfolding could explain many aspects of stress tolerance in that proteins would be further protected from unfolding or misfolding by previously high levels of HSPs.

Competition between ubiquitin and HSP70 provides a possible molecular mechanism. The rescue pathway would prevail in cells containing abundant amounts of HSP70 proteins prior to stress.

Induction of aberrant HSPs by treatment with amino acid analogs does not lead to stress tolerance and even blocks its acquisition, suggesting that functional HSPs are required. It is unclear whether protein synthesis may be a requirement to permit the development of stress tolerance. Most stress tolerant cells show an increase in stress proteins. However, some studies report the development of stress tolerance in the absence of detectable HSP production (Watson et al., 1984). During the period where the development of stress tolerance goes on at its maximum rate, preferential synthesis of HSPs occurs. A quantitative relationship can be established between the concentration of some of the HSPs (particularly HSP70) and levels of stress tolerance, as determined by survival studies. Intracellular levels of HSP70 might, therefore, be used as an indicator of retained stress tolerance (Welch, 1993).

The HSPs are autoregulated. Their presence may turn off further synthesis or lead to destabilization of HSP mRNA resulting in a shorter half-life. The HSPs accumulate rapidly after stress, within 1 minute in cell cultures (Craig, 1985). The return to basal levels may take as long as 8 - 10 days (Emami et al., 1992), differing with the type of inducer. Presumably, tolerance would exist as long as levels of HSPs were elevated.

One widely held hypothesis is that cellular events common to senescent cells, such as oxidant injury and other cumulative forms of cellular damage, might result in a chronic stress response. Older cells with reduced proliferative potential would constitutively express stress proteins at an abnormally high level and consequently mount a less vigorous stress response if exposed to a subsequent stress challenge. In aged rats, a concomitant decline in heat-induced HSP70 mRNA expression has been reported (Blake et al., 1991). The effects that chronic activation and maintenance of a stress response could have on the cell are unclear, since the stress response involves so many vital cell organelles and functions.

Various cellular functions involved in gene expression are impaired by stress, such as rRNA synthesis, pre-mRNA splicing, protein synthesis, and DNA replication, possibly be due to inactivation of crucial enzymatic steps. Changes in phosphorylation, methylation, ubiquitinization and acetylation of histones might be responsible for observed stress-induced changes in chromatin structure. Loss of protein function might also be a consequence of nonspecific protein denaturation from heat *per se* or deleterious modifications due to alterations in cellular metabolism.

With activation of heat shock genes, the expression of most other genes is inhibited as a result of stress, which leads to a perturbation of normal gene expression. Gene expression is also altered in other ways, such as by RNA processing, mRNA stability and translational and transcriptional termination.

Normal protein synthesis was reduced by 70% in cultured cells after treatment with ethanol (Chelbi-Alix and Chousterman, 1992).

Cell growth and proliferation are abruptly arrested in the stressed cell. Mitosis and DNA synthesis are stopped. In homeothermic organisms, the cell cycle is halted throughout the stress response.

Changes in cytoskeletal structure also occur. In nuclei, filaments containing actin are affected; the nucleoli, including the granular ribonucleoprotein components, are transiently damaged, and the assembly and export of ribosomes from these organelles are blocked. The Golgi complex is disrupted and vimentin-containing filaments around the nucleus collapse and aggregate (Morimoto et al., 1990).

Most stress proteins are abundant components of the normal, unstressed cell. After exposure to physiological stress, the increased production (or activation) of the stress proteins occurs as a consequence of perturbations in biochemical pathways in which stress proteins normally participate. Constitutive stress proteins are likely also recruited to perform new tasks that arise because of unique changes occurring in the cell as a result of the stress event. Relatively little is known concerning which metabolic pathways are compromised in the cell experiencing stress or whether the observed alteration is a primary event or a secondary event arising from the change in cellular outlook from maintenance and / or growth to cellular defense.

The majority of HSP70 localizes to the nucleus shortly after its synthesis, within the nucleoplasm, the nuclear matrix, and large amounts within the nucleoli. The nucleolar deposition of HSP70 is correlated with marked alterations in the integrity of the nucleoli, coinciding with the shutdown of rRNA synthesis and new ribosomal assembly. Most of the nucleolar HSP70 is present within the aggregated granular portion, which is involved in the assembly of small ribonucleoproteins and preribosomes. During recovery, HSP70 is observed to exit the nucleolus. HSP70 function within the nucleolus may be to (i) resolubilize denatured preribosomal complexes and/or perhaps facilitate their removal and (ii) facilitate the restoration of normal nucleolar function, in particular ribosomal assembly.

During later recovery, the majority of HSP70 begins to accumulate in the cytoplasm in a strong perinuclear distribution. Much of the cytoplasmic HSP70 is in very close association with the ribosomes and polysomes. Studies with gel filtration and immunological methods indicate that HSP/HSC70 is involved in ATP-dependent interactions with a number of other cytoplasmic and nuclear proteins (Schlesinger et al., 1990).

Subtle changes occur in the organization of the plasma membrane with which HSPs are closely associated during later recovery, but the relative contributions of lipid versus protein thermal transitions and their effect on cell survival are difficult to resolve. Heat shock or other forms of stress effect marked changes in the activity and/or regulation of a number of plasma

membrane components such as (i) a compromise in Na^+ / K^+ ATPase activities, with the net result being an increased level of K^+ and a correspondingly decreased level of Na^+ inside the cell, and (ii) an increase in hexose transport into the cell. These changes could be a reflection of perturbations in the structure/function of membrane components or part of a mechanism by which the stressed cell redirects or shuts down plasma-membrane-mediated events in an effort to protect itself (Morimoto et al., 1990). Binding of certain molecules to their respective receptors is inhibited by heat shock.

The stress response has been observed in all organisms from archeabacteria to mammals. One of the earliest and most profound discoveries resulting from molecular studies was the universality of the stress response. However, the quality and magnitude of the stress response vary widely in nature. Both prokaryotes and eukaryotes respond virtually identically to heat shock and the stress proteins themselves are highly conserved in structure. Craig (1985) reports 60 - 70% homology between prokaryotic and eukaryotic stress proteins.

In general, eukaryotes possess at least 2 copies of most heat shock genes, one under inducible stress regulation and one under constitutive control. Such is the case with the HSP70 family of genes containing the stress induced HSP70 and the constitutively produced HSC70 found normally in unstressed cells. This large multigene family of the eukaryotic genome shares extensive

sequence identity and biochemical properties with the major shock-inducible protein, HSP70. There is a high degree of evolutionary conservation among members of the HSP70 family within a species and between species (Schlesinger et al., 1990) as revealed by the similar size, apparent isoelectric points and tryptic peptide patterns (Wu et al., 1985). The most highly induced stress protein is the 72 kD HSP70, produced in normal, unstressed cells in low or negligible amounts. After stress, HSP70 represents the major translation product of the cell. HSP70 consists of multiple and related isoforms, the exact number depending on the cell type, the agent being used to induce the response, and the severity of the stress treatment (Emami et al., 1991).

In rats, approximately 20 *HSP70*-like sequences have been cloned, but no rat *HSP70* genes have been sequenced. Therefore, the human *HSP70* will be used as a model for rat *HSP70*. Two constitutively synthesized rat *HSC70* cDNAs have been sequenced (Miller et al., 1991; Sorger and Pelham, 1987; O'Malley et al., 1985.).

The expression of HSP70 is regulated in response to diverse conditions that affect cell growth and metabolism ranging from entry into G1 phase of the cell cycle, during development and differentiation, and extending to acute and chronic exposure to physiologically or chemically induced stress conditions. Proper levels of the various HSPs are ensured through a multitude of mechanisms, including gene duplication and by constitutive and inducible expression. Regulation of the *HSP/HSC70* gene can occur at any of the steps

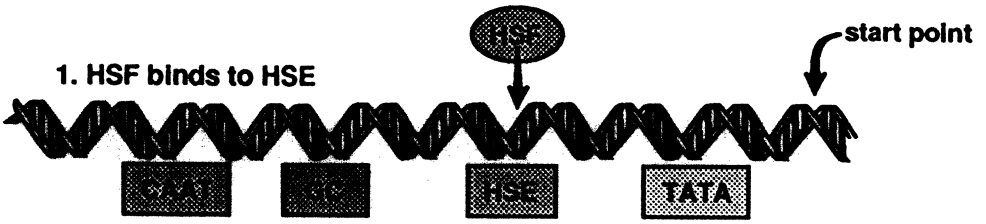
shown in Figure 5, such as activation of gene structure, initiation of transcription, transcript processing, or translation of mRNA. Heat shock genes may be preset in an open chromatin configuration in normal, unstressed conditions with hypersensitive sites at their 5' ends.

Initiation of transcription provides numerous possible control points. Gene expression is rapidly induced at the transcriptional level through protein factor interactions with distinct arrays of *cis*-acting promoter elements located upstream of the human *HSP70* gene. All eukaryotic genes that are transcriptionally induced following exposure to heat shock contain a sequence motif located in the 5'-flanking region referred to as the heat shock element (HSE). The HSE corresponds to inverted multimers of the sequence nGAAn and has been shown to bind with the heat shock transcription factor (HSF). In general, multiple forms of transcriptional regulation are mediated by the presence of additional non-HSE promoter elements, which bind other transcriptional factors (Figure 5).

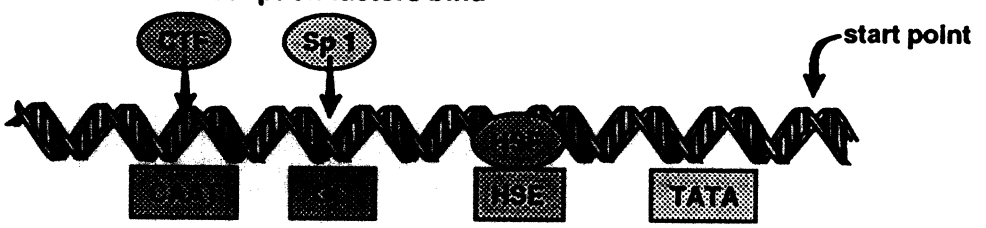
The mechanism by which the heat shock transcriptional apparatus detects heat shock or exposure to chemical inducers of the response is unknown. Benjamin et al., (1992) propose that the DNA binding domain of HSF is masked in unstressed cells by complex formation with HSP70, which would keep DNA binding activity sequestered (Zimarino and Wu, 1987; Mosser et al., 1988). Disruption of such an HSF/HSP70 complex (i.e., during stress) would release HSP70 to associate with unfolded domains of substrate proteins and

Figure 5: Model for transcription of HSP70 gene (Lewin, 1990). HSF binds to the HSE of the promoter, allowing other transcription factors to bind to their respective conserved elements. RNA polymerase binds at the TATA element and begins transcription of RNA from the startpoint. Heteronuclear RNA is transcribed, capped, and polyadenylated to form mRNA. mRNA is released into the nucleus where it is translated by ribosomes into the HSP70 protein.

1. HSF binds to HSE

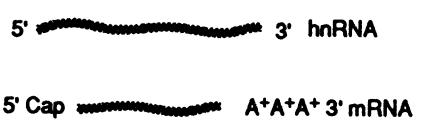


2.Other transcription factors bind



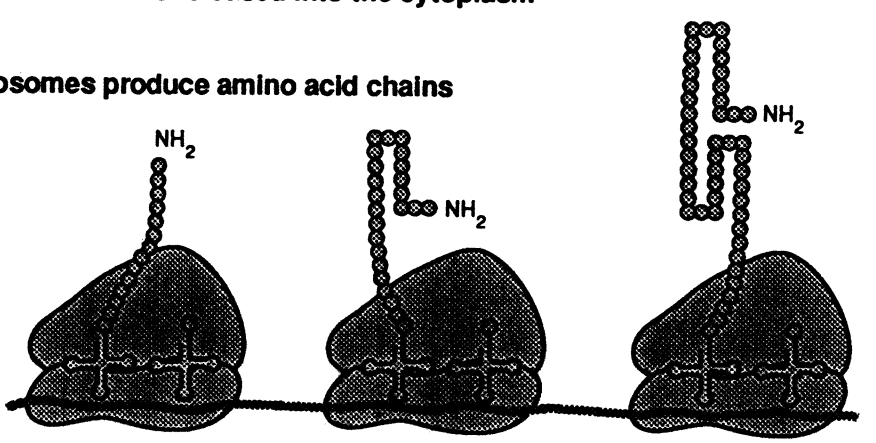
3.RNA polymerase II binds and begins transcription of hn RNA

4. hnRNA is processed to mRNA



5. poly A^+ mRNA is released into the cytoplasm

6. Ribosomes produce amino acid chains

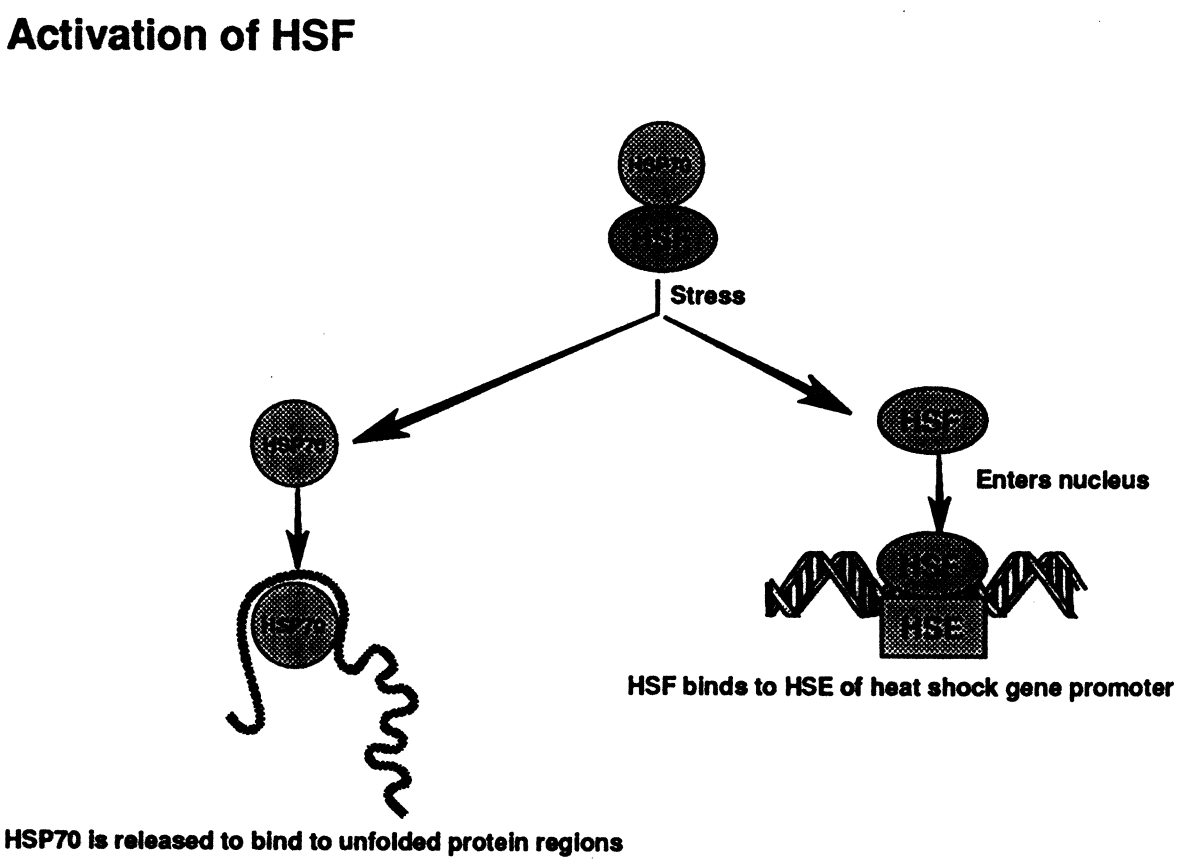


7. Amino acid chains are linked together to form protein

HSF for binding to the HSE, thus resulting in heat shock gene transcriptional activation (Figure 6). The trigger could be the appearance of denatured proteins that would compete with HSF for association with HSP70. HSP70/protein interaction would require ATP to be released. With ATP depletion, HSP70 proteins complexed to nascent or unfolded protein cannot be recycled, thus reducing the free pool of HSP70 available to complex with HSF. ATP depletion may augment intracellular levels of denatured/unfolded protein, thereby increasing demand for HSP70. This HSF/HSP70 association would also explain the positive control autoregulatory aspect of the heat shock response.

The stress-induced form of HSF is phosphorylated. It is likely there are multiple pathways for HSF activation, since activation times vary greatly depending on the inducer of the stress response. HSF is found in the cell for an extended time after the onset of stress. HSP70 is induced by heavy metal ions, as is another protein, metallothionein. The HSE is not utilized for metal-inducible transcription of the metallothionein genes. It is reasonable to suggest that common metal ion-sensitive intermediates in the pathway of transcription activation interact with transcription factors, such as HSF. When HSF activity is activated by inducers other than heat, such as low pH and nonionic detergents, the treatments reduce the thermal denaturation profile for HSF activation. This allows HSF activation to occur under conditions approximating physiological temperatures instead of the extreme temperatures (greater than 43°C) typically

Figure 6: Activation of HSF during stress (Benjamin et al.,1992). In unstressed cells, HSF complexes with HSP70. Under stress, the complex is disrupted due to increased competition for HSP70 by unfolded proteins. HSF binds to the HSE of the gene promoter, activating transcription of HSP70 mRNA. HSP70 is free to bind to unfolded protein regions.



required to activate HSF *in vitro*. The biochemical events involved in HSF activation may require altered protein conformation (Schlesinger et al., 1990).

The human *HSP70* promoter is composed of at least two functional domains (Wu et al., 1985). The proximal domain which extends to -68 is required for basal expression and responds to signals that activate cell growth and transcriptional transactivation. The distal promoter contains the HSE which is necessary to respond to multiple forms of stress induction. Even further upstream of -120 are additional basal elements that are redundant to sequences in the proximal promoter.

Basal expression of the human *HSP70* gene does not depend on the HSE. The promoter sequences necessary for heat shock and metal ion induction of the human *HSP70* gene are located in the distal domain of the promoter extending from -107 to -68, which contains matches for elements of the HSE. The minimum sequence requirement for heat shock, heavy metal and amino acid analog activation of *HSP70* transcription is consistent with the definition of the minimal functional HSE. Stress signals are mediated in part through interactions between specific protein factors and a complex array of sequence elements located within 100 base pairs of the site of transcription initiation, such as a CCAAT element at -68, a GC element at -45, and a TATA element at -28. HeLa cells contain two HSE-specific DNA binding activities, one found in unstressed cells, the other in stressed cells, each with distinct electrophoretic mobility on gel shift assays. Those found in extracts from

stressed cells presumably correspond to the HSF. Both HSE-binding activities bind to DNA with nearly identical nucleotide specificities (Hunt and Morimoto, 1985).

Regulation can also occur posttranscriptionally by the increased stability of heat shock mRNAs during stress or translationally by control of both initiation and elongation of protein synthesis. HSP70 mRNAs, extremely unstable at normal temperatures (unstressed conditions), are stabilized under stress (Craig, 1985). Heat shock messages are translated with high efficiency; preexisting mRNAs are translationally repressed, leading to decreased competition for translation.

The mRNA for human HSP70 contains no introns (Wu et al., 1985; Hunt and Morimoto, 1985). The gene sequence of *HSP70* contains an open reading frame that is transcribed uninterrupted in mRNA from 2.6 kb to 3.53 kb (Miller et al., 1991). The lack of intervening sequences may be significant in a gene that is rapidly activated at the transcriptional level in a wide range of cell types. Unlike *HSP70*, the gene sequence for the constitutive HSC70 contains 8 introns which may differ in length, but are of the same position relative to the protein coding region. The 5' and 3' boundaries of each intron are in good agreement with the consensus sequence for exon-intron splice junctions of other eukaryotic genes. The *HSC70* gene encodes mRNAs reported from 2.3 - 2.5 kb in various studies, but always smaller than mRNAs for HSP70 (Miller et al., 1991; Blake et al., 1991).

The mammalian genome contains many *HSC70*-like genes or pseudogenes. In rats, the majority of *HSC70*-related sequences (approximately 25 in all) are processed pseudogenes. Sorger and Pelham (1987) suggested that HSC70 in rats is encoded by either a single gene or by a small family of very closely related genes. The abundance of *HSC70* pseudogenes is probably a consequence of the high concentration of HSC70 mRNA in early mouse (and presumably rat) embryos. Numerous intronless *HSC70* pseudogenes could be remnant copies of *HSC70* reverse transcripts, while the presence of multiple stop codons suggest that these copies are not functional.

As is the case with *HSP70*, transcription of *HSC70* allows numerous possible control points. Genomic *HSC70* sequence contains three putative binding sites for transcription factor Sp1: a TATA motif, two inverted CAAT boxes and two regions resembling an HSE and a metal-response element upstream of the transcription start site. Dworniczak and Mirault (1987) found that HSC70 could be induced two-fold by hyperthermia, additional stimulation of already very high constitutive expression of this gene. Copies of HSE are present in promoters of mammalian *HSC70* genes which also have significant heat shock inducibility. This may support high constitutive transcription of HSC genes even under adverse conditions and avoidance of transcriptional inhibition experienced by other cellular genes after severe heat shock (Zafarullah et al., 1992; Craig, 1985). Sorger and Pelham (1987) report levels of HSC70 mRNA

are about 5 times higher in rapidly growing tissue-culture cells than in cells whose growth has been arrested by serum starvation.

The HSC70 mRNA transcript is approximately 2.3 kb (Brown and Rush, 1990) and has an open reading frame of 1938 nucleotides which encode a protein of 70899 daltons. At the nucleotide sequence level, the *HSP70* and *HSC70* genes display a homology of 74% while the homology increases to 81% at the predicted amino acid sequence level with higher divergence at the carboxy terminal regions of the proteins (Dworniczak and Mirault, 1987). In heat stressed cells, there is no decrease in HSC70 mRNA levels as compared with unstressed cells. HSC70 can comprise up to 1% of the protein in growing cells (Sorger and Pelham, 1987).

Stress Proteins and Ethanol

Early studies with HSPs noted the phenomenon of stress tolerance. An analog to heat was sought that would confer subsequent thermotolerance without damaging the cells as heat did. Thus, Li and Hahn (1978) reported that ethanol induced the stress response by increasing survival of cultured cells.

Ethanol within a cell denatures proteins and fluidizes membranes. Ethanol is oxidized to acetaldehyde mainly in the liver via alcohol dehydrogenase. Acetaldehyde, in turn, is further oxidized by aldehyde dehydrogenase to form acetate (Lin et al., 1988). Acetaldehyde has been implicated in a number of actions of alcohol as well as hepatotoxicity due to

alcohol consumption. Acetaldehyde can bind to plasma proteins, albumin, erythrocyte membrane proteins, tubulin, hepatic proteins, hemoglobin and critical lysine residues of a number of enzymes.

Cultured cells exposed to 6% ethanol in culture media were shown to have increased survival following exposure to heat (Li and Hahn, 1978; Li et al., 1980). Further analysis with electrophoretic gels determined that cells exposed to 6% ethanol in culture media synthesized proteins of approximately 70 kD in greater concentrations than cells not exposed to ethanol (Li, 1983). Lower concentrations of ethanol (4%) increased concentrations of 70 kD proteins only when combined with heat. The two forms of stress acted synergistically since neither alone was able to produce the stress response. Monoclonal antibodies were used to show that the 70 kD proteins produced were HSPs (Hahn et al., 1991).

Ethanol may affect cells by increasing the levels of 2',5'-oligoadenylates, potent inhibitors of protein synthesis (Chelbi-Alix and Chousterman, 1992) in cultured cells. 2',5'-oligoadenylates increased following exposure to 7.5% ethanol in the culture medium. Heat shocked cells also showed increased 2',5'-adenylate synthetase during recovery after heat shock.

Expression of 70 kD HSP/HSC following exposure of cultured cells to 6% ethanol in culture media depended on new RNA synthesis (Rodenhiser et al., 1986). Nakamura et al., (1991) reported accelerated transcription of both the inducible 72 kD HSP70 and constitutive 74 kD HSC70 after ethanol was added

to culture media. Both studies used Northern blotting to assess changes in mRNA.

The concentrations of ethanol needed to induce the stress response in cultured cells at short exposure (5-6%) are physiologically unfeasible (Walsh and Crabb, 1989). Lower concentrations of ethanol at short exposures did not activate the stress response. They suggested, however, that the heat shock response may be induced at low levels of ethanol for longer periods *in vivo*, or in tissues directly exposed to alcoholic beverages. This would be consistent with the low toxicity of ethanol and the long periods of time required for the development of clinically significant alcohol-induced tissue injury. In support of this theory, liver tissues obtained by routine surgical pathology methods from patients who were "heavy drinkers" for at least 10 years and controls who were not, were exposed to polyclonal antibodies specific for the inducible HSP70. HSP70 immunoreactivity was seen only in the experimental group. The effect of duration or degree of alcohol exposure on HSP70 immunoreactivity in "heavy drinkers" could not be assessed. However, the histomorphologic diagnosis of alcoholic liver disease ranging from alcoholic hepatitis to cirrhosis was supported by clinical history.

In contrast, Miles et al., (1991; 1992) reported that ethanol led to an increase in the constitutive HSC70, rather than the inducible HSP70 in hybrid neuroblastoma x glioma cultured cells as measured by Northern blot analysis. The concentrations of ethanol used (50-300 mM) are similar to those seen in

actively drinking alcoholics and produced significant increases in HSC70 mRNA and protein. Probes and antibodies used were specific for either the constitutive or inducible forms, with no appreciable cross-hybridization or reactivity. Lower concentrations (50 mM) were effective in cells treated for longer periods of time (6 days). Withdrawal of ethanol resulted in a return of HSC70 mRNA to near basal levels. However, cells continuously treated with ethanol showed elevated levels of HSC70 even after weeks of treatment. Immunoreactivity for HSP70 demonstrated undetectable amounts in control or ethanol-treated samples. Alcohols of higher chain length and greater lipophilicity were more potent in producing intoxication and dependence in animal models than ethanol. Induction of HSC70 mRNA was proportional to the chain length of the aliphatic alcohol, suggesting that lipophilic rather than osmotic properties of ethanol were involved in HSC70 induction. Northern blot analysis with HSP-specific probes failed to detect any HSP70 mRNA in control cells or in cells exposed to up to 300 mM ethanol for 1 hour to 6 days. Thus, they concluded that low levels of ethanol did not induce the typical stress response (the induction of HSP70), but rather represented an alcohol-specific response. HSC70 mRNA induction occurred over a concentration range and time course that correlated well with animal models of alcoholic tolerance and dependence. Induction of stress proteins by several agents generally occurred more rapidly than the induction of HSC70 by ethanol seen here. The data suggested that at least some of the ethanol-induced alterations in cell surface

receptor abundance and internalization may have been post-translational effects secondary to changes in HSC70 expression.

Zatloukal et al., (1988) investigating the HSP response in normal and diseased hepatocytes, reported ethanol concentrations of 4, 6, and 8% in culture media actually reduced synthesis of a 74 kD protein (HSC70) and determined that no new protein or RNA synthesis was necessary to the production of stress tolerance. Further, they predicted that pathological alterations of cells and organs could modulate the stress response, since diseased hepatocytes did not increase amounts of HSPs after ethanol exposure as normal hepatocytes did.

In situ Hybridization of HSP/HSC70

In studies of ethanol and HSPs, cell survival was commonly used to assess tolerance. Gel electrophoresis was used to assess changes in protein expression and mRNAs following induction by ischemia, heat, etc. Antibodies specific for inducible HSP70 or constitutive HSC70 allowed more accurate interpretation of the gels (Satoh et al., 1992; Sharp et al., 1991; Kitagowa et al., 1991). Riboprobes and cDNA probes were developed to assess gels and blots (Tremblay et al., 1992; Brown and Rush, 1990; Manzerra and Brown, 1990). The first probes could not distinguish between HSP70 and HSC70. However, oligonucleotide probes developed recently are specific for either HSP70 or HSC70 mRNA (Miller, et al., 1991) with no cross reactivity. As has already

been noted, HSP70 and HSC70 share extensive sequence homology. These probes have been used extensively for *in situ* hybridization histochemistry.

The method of Wisden et al., (1991) allows the detection of mRNA for a specific protein within intact tissues at both regional and cellular levels, using a labeled nucleic acid probe of complementary sequence to a portion of the mRNA. Short synthetic oligonucleotide probes are ideal due to their specificity and their ability to penetrate tissue easily. Oligonucleotides are very useful when probes are required that cleanly distinguish closely related or alternatively spliced transcripts. This method is minimal in terms of the number of procedures required compared to many other protocols, and the sensitivity of detection appears to be at least comparable to, if not greater than, that obtained with other methods. For *in situ* hybridization, a 1:30 molar ratio of probe to isotopic base is recommended in the labeling reaction.

The oligonucleotide probe sequences used here, specific for either the inducible HSP70 or the constitutive HSC70 mRNA, were determined by Miller et al., (1991) by RNA isolation from female Sprague-Dawley rats (Table 2).

Table 2: Sequences of probes specific for HSP70 or HSC70 (Miller et al., 1991)

HSP70	5' ATCTCCTTCATCTTGGTCAGCACCATGGAC 3'
HSC70	5' ATGCCTGTGAGCTCAAACTTCCCAAGCAGG 3'

Their strategy relied on comparative sequence data of human, *Drosophila*, mouse and rat DNA to determine unique regions of rat inducible and constitutive 70 kD HSP. A 30-nucleotide region with 50% GC base content was identified for HSP70. The third nucleotide positions of HSC70 codons contain a higher percentage of the bases A and T than the third nucleotide positions of HSP70 codon. An oligonucleotide synthesized from the region of the rat HSC70 coding sequence corresponding to the region of the HSP70 probe would have a GC base composition of only 30%. Therefore, nucleotides 1,413 - 1,442 were chosen for the HSC70-specific probe. This region of the rat HSC70 sequence was mismatched by two nucleotides to human HSC70 sequences and by four nucleotides to mouse HSC60. Neither of these oligonucleotide probes was sufficiently similar to the corresponding regions in a fourth member of this gene family, the glucose-regulated protein 78 for cross-hybridization to occur. Probe sequences were checked against known sequences in both Genbank and EMBL Nucleic Acid Databases. The QUEST and FASTdb programs in the Intelligenetics Suite (Bionet) were utilized to search for sequences identical to or similar to the selected probes. The probe sequences also met the requirement of an ideal GC/AT ratio of approximately 50% or more. Oligonucleotides with large numbers of A and T residues will be less stable in forming DNA/RNA hybrids. High GC content (>65%) may cause nonspecific binding since the thermal stability of the probes will be much greater. The HSC70-specific oligonucleotide probe identified a 2.55 kb mRNA

in cerebellum and cerebral cortex of both control and experimental rats. The HSP70-specific oligonucleotide probe identified a 3.05 kb mRNA and a 3.53 kb mRNA in cerebellum and cerebral cortex of heat-shocked and amphetamine-treated rats, but not in control rats. Quantification by comparison with 18S rRNA levels demonstrated that both HSP70 and HSC70 mRNA levels increased following each treatment and were higher in cerebellum than in cerebral cortex. The results suggested that both the transcription and turnover of HSP70 mRNAs differed between cerebellum and cerebral cortex.

A very similar oligonucleotide probe specific for HSP70 was constructed by Nowak (1990), differing by one base. Nowak confirmed its specificity for the inducible HSP70 mRNAs in mouse brain and liver and other authors have since employed this probe for *in situ* hybridization (Blake et al., 1991). Brown and Rush (1990) showed that probes containing parts of the nontranslated regionsof the human HSX70 and HSC70 sequences hybridize specifically to rodent HSP70 and HSC70 mRNAs, respectively.

Masing et al., (1990) recommended the use of *in situ* hybridization histochemistry in the study of HSPs to obtain evidence (i.e., detect mRNAs) for the protein actually synthesized in that cell type, rather than imported from another cell. Antibodies detected only the final protein, which may or may not have been synthesized in the cell type in which it was found. Previous investigations utilizing *in situ* hybridization histochemistry on brain tissues have induced transcription of HSP70 mRNA with hyperthermia (Manzerra and Brown,

1992; Masing and Brown, 1989; Masing et al., 1990) and ischemia (Kawagoe et al., 1992; Nowak, 1991), but the effects of ethanol on the constitutive transcription of mRNA for HSC70 or the induced transcription of mRNA for HSP70 have not been analyzed *in vivo* with *in situ* hybridization histochemistry.

In the studies mentioned above, HSP70 was distinguished from HSC70 either by using a specific probe for mRNA of either HSP70 or HSC70 or by using a probe which hybridized to both mRNAs and separating the mRNAs by size electrophoretically. The inducible HSP70 mRNA was absent or present in negligible amounts in control animals in all areas and induced in experimental subjects in the following areas: fiber tracts throughout the forebrain and cerebellum, Bergman glial cells in the Purkinje layer, oligodendroglial cells in deep white matter and granule neurons, lateral geniculate body, medial habenula, ventral thalamic nuclei, medial amygdaloid, arcuatal nuclei, ventromedial hypothalamus, caudate, dorsolateral striatum, piriform, entorhinal and neocortical regions, hippocampal CA1-CA4 neuronal cells, hippocampal CA3 pyramidal cells, and dentate gyrus granule cells. The loss of hybridization signals for the induced HSP70 mRNA took as long as 7 days after the stress event.

HSC70 mRNA was found in tissues from both control and experimental animals in relatively the same quantities. The amount of hybridization signal has been measured only on a relative scale in the literature. Hybridization

signal was detected in Purkinje neurons in the Purkinje cellular layer and granule cell layer neurons, but not the cerebellar molecular layer in any neuronal or glial cell type, nor in the pia mater. Constitutive expression was also exhibited in hippocampal neurons (Manzerra and Brown, 1992; Masing and Brown, 1989).

Rationale

Ethanol causes major changes in cells as an oxidative agent: fluidizing membranes, altering receptors, and neurotransmitters. With prolonged exposure to ethanol, tolerance to the intoxicating effects of ethanol develops, with further changes to cell receptors, etc. Thus it is predicted that responding for ethanol will increase after the administration of liquid diet containing ethanol. Cellular changes due to ethanol are likely to change levels of stress proteins. *In situ* hybridization was performed to test the hypothesis that mRNAs for HSC70 and HSP70 would be different in tissues from animals fed liquid diet with ethanol from tissues in animals fed a similar liquid diet without ethanol. Specific oligonucleotide probes were used to distinguish between mRNA for the constitutive HSC70 and the inducible HSP70.

CHAPTER 2

MATERIALS AND METHODS

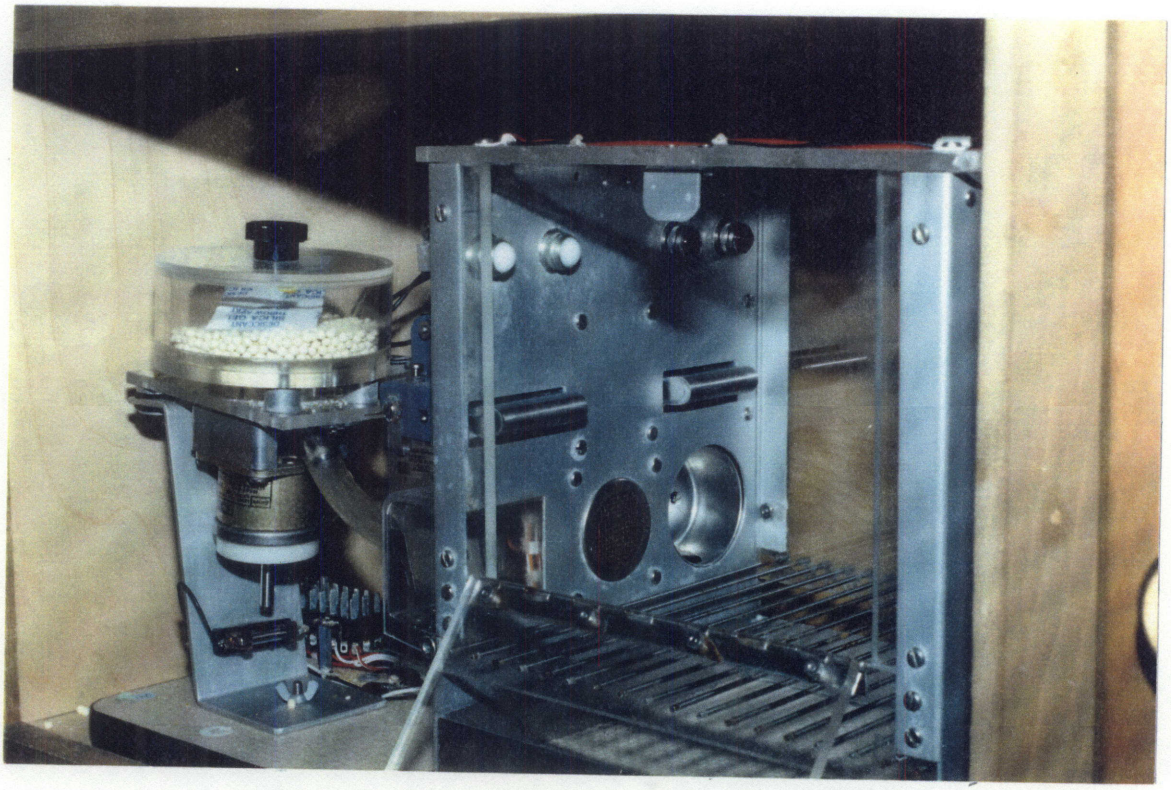
Animals

Male Fischer 344 rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed individually in a vivarium at constant temperature (21 - 23°C) with 12 hour light / 12 hour dark cycle. Prior to the experiment, Purina rodent chow and water were constantly available in the home cage. When the animals were approximately 3 months old and reached an average weight of 250 g, experiments were begun. Nonethanol subjects were kept in home cages with *ad libitum* food and water until the second liquid diet administration.

Equipment

Test chambers measured 21 x 24 x 21 cm with parallel steel bars for the flooring and a removable tray underneath (Figure 7). White house lights, which were activated throughout the sessions, were mounted in the top of the boxes. The left wall comprised the subject interaction area. Two levers were mounted 8 cm from the floor on either side of a center mounted speaker, which was not used in this experiment; lever lights, mounted above the levers, were lit when the levers were activated. Only the right lever, with red lever lights, was activated during these sessions. A square alcove, located on the far left at floor

Figure 7: Animal training and test chambers. Left lever pressing allows presentation of a food pellet in the square alcove. Right lever pressing allows dipper presentation in the round alcove on the right.

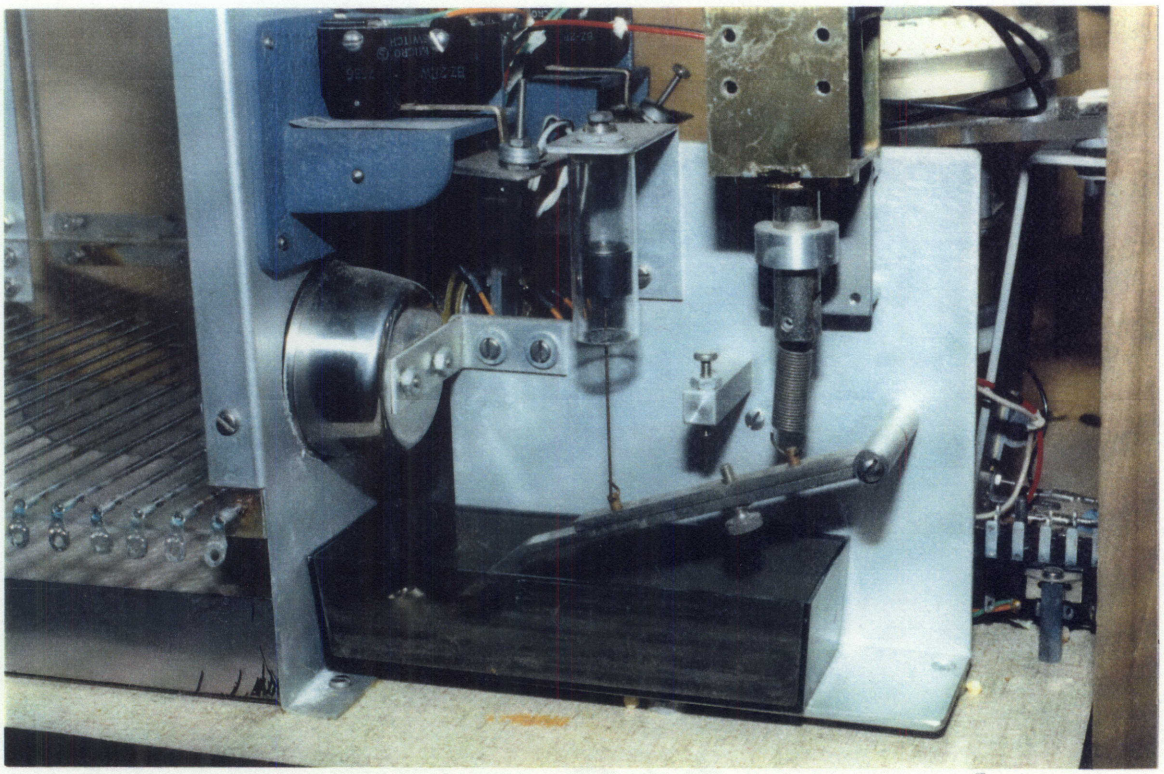


level, delivered food pellets via an automated feeder that clicked when the feeder was advanced and a pellet was available. A round alcove with a circle cut into the bottom for presentation of a dipper containing 100 µL of solution was to the far right at floor level (Figure 8). A loud pop sounded when the dipper was raised. The boxes were run with a 386 microcomputer, an automated program and an LVB interface (Med Associates, East Fairfiled, VT).

Materials

Sucrose was obtained from Sigma Chemical Co. (St. Louis, MO) and ethanol from Midwest Grain Products (Perkin, IL). Ingredients for the liquid diet were purchased from ICN Biochemicals (Plainview, NY; casein, vitamins and minerals), Sigma Chemical Co. (St. Louis, MO; methionine, xanthum gum, choline and dextrin), and local food vendors (corn oil). For *in situ* hybridization, diethylpyrocarbonate (DEPC), poly-L-lysine hydrobromide, paraformaldehyde, phosphate-uffered saline, oligo (dT) cellulose, dithiothreitol (DTT), salmon sperm DNA, formamide, dextran sulfate and Denhardt's solution were all purchased from Sigma Chemical Co. (St. Louis, MO). Terminal deoxynucleotidyl transferase with 5X buffer and 20X Standard Sodium Citrate (SSC) were purchased from Gibco BRL (Grand Island, NY). Sodium heparin was from United States Biochemical Corp. (Cleveland, OH). Synthetic oligonucleotide 30-mer probes for *in situ* hybridization were purchased from National Biosciences Inc. (Plymouth, MN). Radiolabeled dATP was from New

Figure 8: Dipper apparatus. When right lever is pressed appropriately, the dipper arm, containing 100 µL of solution in the well, is presented in the round alcove on the right of the test chamber.



England Nuclear (Boston, MA). All other chemicals were of the highest grade commercially available.

Self-Administration Training

Rats were food and water deprived for 24 hours prior to the first shaping session. The behavior shaping, or training session, employed a fixed ratio (FR) of 1 lever press on the right lever for 1 dipper presentation or reinforcement. The FR1 schedule was maintained for the entire program. In addition, the first 25 presses on the right lever resulted in the release of 1 food pellet in the square alcove on the left and dipper presentation for 10 seconds in the round alcove on the right. Subsequent right lever presses resulted in dipper presentation only with no food reinforcement. The solution was 20% sucrose (w/v, 20 g/100 ml) and the session ran overnight. Several rats responded favorably. These rats were run again on another shaping program with 5 reinforcements each for FR1, 2 and 3 and unlimited FR4 dipper presentations of 20% sucrose solution for 3 seconds. This final shaping program ran 4 hours.

Training sessions were begun with 3 second presentations of 20% sucrose on an FR4 schedule for 30 minutes with *ad libitum* food and water available in the home cage. A total of eight rats had sufficiently high lever pressing to be included in further training. The remaining 7 rats were kept in home cages as nonethanol subjects for the *in situ* hybridization histochemistry.

The sucrose concentration was reduced and ethanol gradually introduced according to the schedule in Table 3. The most stable lever pressing occurred when 5% sucrose was included in the ethanol solutions, despite repeated attempts to lower and/or eliminate the sucrose from the solutions. Animals would only respond by lever pressing for solutions without ethanol if they had been fluid deprived for at least 24 hours. To test a range of ethanol concentrations without sucrose (0.625, 1.25, 2.5, 5, 10 and 20% ethanol, v/v), animals were fluid deprived for 24 hours with food available. Test sessions were 30 minutes long with 12 hours between sessions. Lower concentrations of ethanol were randomly ordered, but the highest concentration was run last.

Table 3: Training to Self-Administer Ethanol

Percent Ethanol	Percent Sucrose	Number of Sessions	Mean mg/kg	Mean Lever Presses
			Sucrose	
0	20	6	3015	167
0	10	2	1763	200
			Ethanol	
2	10	4	371	241
5	10	3	607	166
5	5	3	788	216
10	5	4	1326	189
10	0	4	623	95

Water was available in the home cage for 1 hour after the test session and food was always available in the home cage. After the final test, animals were allowed to rest for a day before the liquid ethanol diet was administered.

Ethanol Diet

Each liter of this diet contained an aqueous suspension of micropulverized casein (42 g), L-methionine (0.6 g), vitamin mixture (2.1 g), mineral mixture (7.3 g), xantham gum (3 g), choline chloride (0.4 g), and corn oil (10.5 g). The ethanol diet also contained sucrose (25 g), dextrin (36 g), ethanol (52.8 mL) and saccharin (0.215 g). The non-ethanol diet contained dextrin (114.75 g) and sucrose (25 g). Dextrin and ethanol concentrations varied in concert to provide the same caloric and nutritional content in both formulations. Fresh 100 mL aliquots of the liquid diet were delivered into graduated glass feeding tubes (Richter tubes, Harvard Apparatus, Millis, MA) and placed in the home cages daily. While the diet was administered, no water was available. 100 mL aliquots of liquid ethanol diet were administered to only the 8 ethanol test animals for 3 days. 24 hours after the final diet presentation, animals were tested again at a low concentration of ethanol. No animals responded favorably. The animals were fluid deprived for 24 hours and another series of tests conducted. After the final test, animals were given free food and water and were allowed to rest in their home cages for several weeks.

Finally, liquid diet containing ethanol and a calorically balanced liquid diet without ethanol were presented to the appropriate subjects after 24 hours of food and fluid deprivation. 100 mL aliquots of diet were given to the animals each day for 2 weeks.

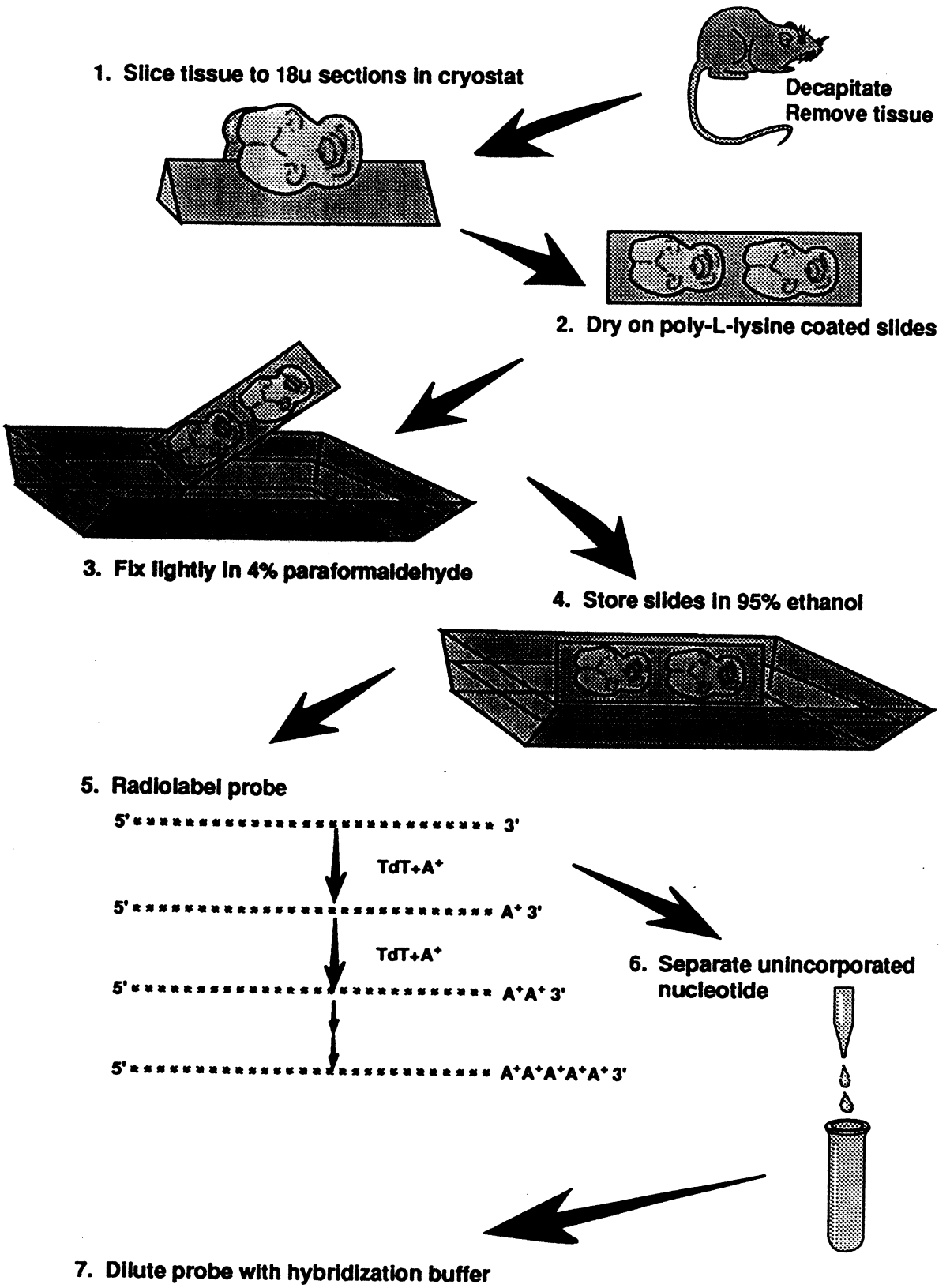
In situ Hybridization Histochemistry

All solutions used prior to and during the hybridization step were rendered sterile and free of contaminating ribonucleases (RNase) by adding DEPC (1 mL/L). Glassware was wrapped in aluminum foil and baked at 180°C for 4 hours. All other equipment was autoclaved.

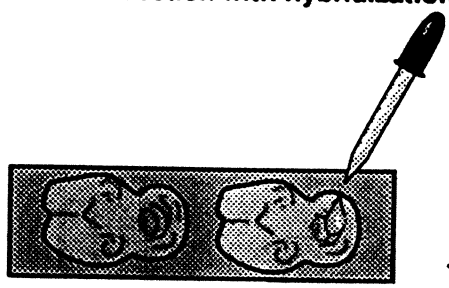
Animals were killed by decapitation (Figure 9), 24 hours after the last diet presentation. Tissue from brain, liver, kidney, thigh muscle, heart, and gastrointestine were dissected fresh (not perfusion fixed) and quick frozen on aluminum foil resting on dry ice. Tissues were then wrapped in alternating layers of aluminum foil and Parafilm and stored at -80°C until ready for use. The tissue was transferred to the cryostat chamber at -20°C to equilibrate, mounted onto a cutting block with cryoglue (Tissuetek, Fisher Scientific Products) and sectioned at 18 microns. Sections were thaw mounted onto poly-L-lysine-coated slides and allowed to air dry several hours.

A rack of dry sections was transfered into ice cold 4% paraformaldehyde for 5 minutes in a very light fixation step sufficient to retain RNA in the section. The rack of sections was transfered into 1X PBS for 1 minute to briefly rinse,

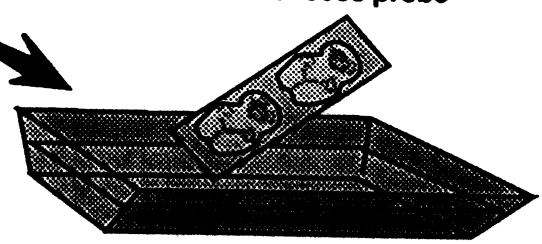
Figure 9: *In situ* hybridization procedure.



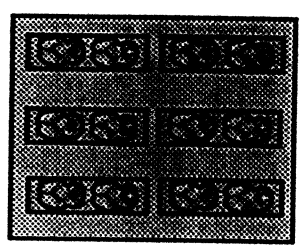
8. Cover section with hybridization buffer and radiolabelled probe



9. Wash to remove excess probe

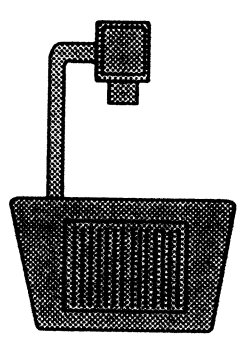


10. Expose to xray film with radioactive microscales



11. Develop film

12. Determine radiolabelling of mRNA in tissue sections with automated image analysis system



light box

computer

then into 70% ethanol for 2 - 3 minutes to dehydrate and finally into a storage box containing 95% ethanol. The airtight storage box, with 95% ethanol covering all sections, was stored at 4°C in a cold room until required. Just prior to hybridization, sections were removed from the 95% ethanol and dried.

Hybridization buffer was prepared with 25 mL deionized formamide, 10 mL 20X SSC, 2.5 mL of 0.5M sodium phosphate, pH 7.0, 0.5 mL of 0.1M sodium pyrophosphate, 5 mL 50X Denhardt's solution, 2.5 mL of 4 mg/mL acid/alkali hydrolysed salmon sperm DNA, 1 mL of 120 mg/mL heparin, and 5 g dextran sulfate. Volume was adjusted to 50 mL with DEPC treated water. The solution was vortexed occasionally over several hours to dissolve the dextran sulfate, then stored at 4°C wrapped in aluminum foil. The solution was not boiled prior to use.

Oligonucleotide probes, specific for mRNA of either HSC70 or HSP70, were 3' end labeled with terminal deoxytransferase using 5'- α -[^{35}S] dATP (1200 Ci/mmol), with an average addition of 30 AMP residues. 2 µL of 5X tailing buffer, 1 µL 30-mer oligonucleotide (3 ng/µL, 0.3 pmol/µL), 5.2 µL of DEPC treated water, 1.5 µL of 5'- α -[^{35}S] dATP (1200 Ci/mmol, 12.5 µCi/µL), and 1.3 µL of terminal deoxytransferase (15 Units/µL) were added to the microcentrigfuge tube and mixed gently by pipetting up and down, using care not to introduce air bubbles which would inhibit the enzyme. The solution was incubated at 37°C for 5 minutes; then the reaction was stopped by adding 39 µL of DEPC treated water.

Unincorporated nucleotides were separated from labeled probe by a gravity column procedure using NICK columns (Pharmacia). The 50 µL of probe solution was pipetted onto the top of the column and 400 µL of sterile water was added and allowed to enter into the gel bed. The sample was collected in a sterile 1.5 mL microcentrifuge tube under the column. Purified sample was eluted with 400 µL sterile water. 20 µL of 1 M dithiothreitol (DTT) was added to the eluate to prevent crosslinking of sulfur residues in the ^{35}S-labeled probes. Probes were diluted in hybridization buffer to a concentration of 1 pg/µL.

Sections were removed from the 95% ethanol storage and air dried for approximately 30 minutes. Slides were laid horizontally in a plastic slide tray. 100 µL of the appropriate probe/hybridization solution was applied to tissue sections. A Parafilm coverslip was gently lowered over the drop of probe/ hybridization solution to spread the liquid smoothly. Any air bubbles were removed by gently pressing with forceps. Paper towels were saturated with 50% formamide / 4X SSC and placed in the bottom of a plastic, well sealed container (Rubbermaid, USA). Several slide trays containing the hybridizing sections were stacked on top of each other allowing air to circulate. The sealed container was incubated overnight at 42 °C in a water bath.

Slides (with Parafilm coverslips on) were transfered into a sterile petri dish containing 1X SSC at room temperature. Coverslips were gently dislodged with blunt ended forceps. A rack of slides was transferred into 250 mL

prewarmed 1X SSC at 55 °C (in a gently shaking water bath) and washed for 30 minutes, then transferred through a very brief (several seconds) series of room temperature rinses in 1X SSC, 0.1X SSC, 70% ethanol, and 95% ethanol. Sections were air dried and slides exposed to Kodak XAR-5 x-ray film at room temperature for 28 days.

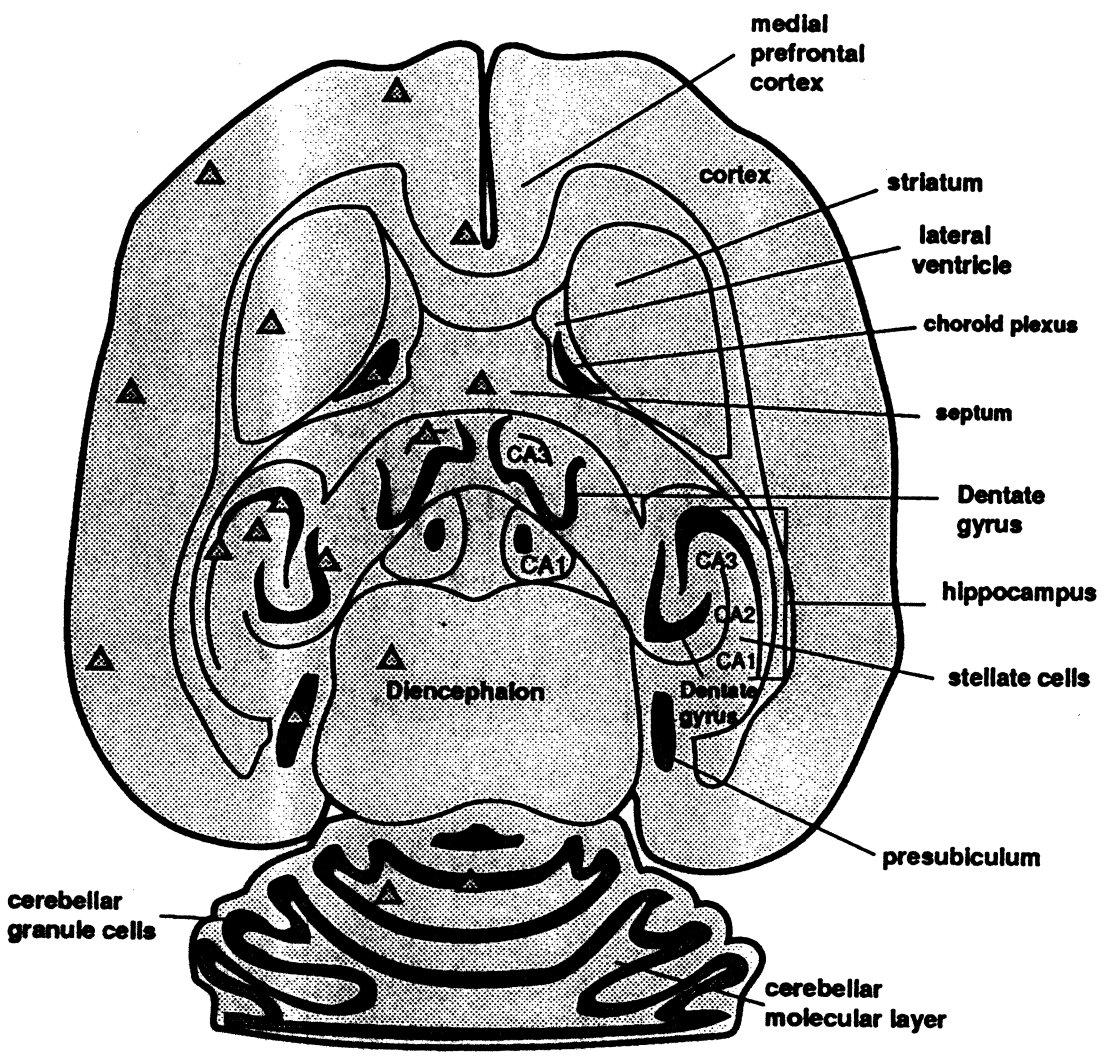
Film was developed as recommended by the manufacturer in an automated film developer. For quantitative analysis of autoradiograms, ^{14}C microscales (Amersham) were exposed along with the sections due to the similar maximum β energy values between ^{14}C (0.156 MeV) and ^{35}S (0.167 MeV).

Films were analyzed using MCID (MicroComputer Imaging Device, Imaging Research Inc., Brock University, St. Catherines, Ontario, Canada). After the equipment was calibrated for light levels, the ^{14}C microscale standards were scanned. The developed film showed standard concentrations of 5.1, 10.1, 30.5, 61.0 and 103.0 μCi/mg tissue. The program automatically computed the standard curve for these concentrations by measuring relative optical density for each concentration. Next, the film corresponding to each tissue section was scanned with measurements randomly taken for each region listed in Table 4, yielding a value of μCi radiolabel/mg tissue. In gastrointestinal, heart, liver and skeletal muscle tissue, samples were taken randomly throughout the section. For kidney tissue, samples were taken from cortex, papilla and medulla. Brain regions sampled are shown in Figure 10.

Statistical Analyses

Analysis of variance was used to compare response levels for pre- and post-chronic dose effect curves as well as the pattern of responding throughout the test sessions as divided into 5 minute time bins. Radiolabeling data were not normally distributed, so nonparametric statistics were used. Analysis of variance on ranked data was used to interpret the results of *in situ* hybridization within each region and Wilcoxon ranked sums test was employed to compare radiolabeling of the same probe in ethanol and nonethanol tissues.

Figure 10: Brain regions sampled for radiolabelling. Brain sections ranged from Bregma -4.74 to Bregma -3.10 (Paxinos and Watson, 1988). Therefore, all regions were not represented in all sections. Table 4 indicates the number of samples taken for each region in each group type.



CHAPTER 3

RESULTS

Behavior Shaping

Two shaping programs were attempted before success was attained with the program outlined in the procedure. In the first, lever pressing at a fixed ratio (FR) of 1 press per 3 second dipper presentation was continued for 10 dipper presentations to train the animals to lever press for 20% sucrose solution. The FR increased to 2 for 10 reinforcements, then 3 for 10 reinforcements and finally unlimited reinforcements at FR4. The session ran overnight. Only 1 rat of 15 achieved FR4. Therefore, a second shaping program was attempted using a 5 second dipper presentation and 15 reinforcements for FR1, FR2 and FR3. Again, the solution was 20% sucrose and again the FR4 schedule had unlimited reinforcements. No more rats achieved FR4. Finally, the paradigm listed in the procedure allowed successful training with 8 rats.

Self-Administration Training

Samson's technique of sucrose fading was used to train rats to lever press for ethanol according to the schedule listed in Table 3. After initial training to 10% ethanol, responding dropped significantly for 6 of the 8 rats with less than 50 lever presses per day on days 3 and 4. Sucrose was added in the

training solution for several days until responding was at least 100 lever presses for all 8 rats. When sucrose was removed from the ethanol solution, even at ethanol concentrations as low as 2.5%, responding dropped below 50 lever presses for several rats, despite repeated manipulations of both ethanol and sucrose concentrations. Stable lever pressing was maintained with 5% sucrose included with ethanol concentrations up to 10%. To test for responding for ethanol alone, animals had to be fluid deprived.

Ethanol Behavioral Testing

The animals were fluid deprived with free feeding for 24 hours prior to testing with various levels of ethanol as shown in Figures 11 and 12. The testing sessions lasted 30 minutes. Highest lever pressing occurred at 1.25% ethanol, with a mean of 208 lever presses for the 8 rats (Figure 11). Mean lever pressing at 2.5% ethanol was 123, 5% ethanol was 195 and 10% ethanol was 125. Ethanol intake rose with the solution concentration (Figure 12). Mean ethanol intake (g/kg) was 0.18 at 1.25% ethanol, 0.29 at 2.5% ethanol, 0.74 at 5% ethanol and 0.86 at 10% ethanol, despite fluctuations in lever pressing. Liquid diet containing ethanol was administered for 4 days with mean consumption of 71, 86, 60, and 100 mL of diet per day. Consequently, the mean ethanol intake for each day was 7.5, 9.1, 6.3, and 10.6 g/kg (Figure 13). Responding for ethanol increased after the ethanol liquid diet was administered for 4 days. (Figures 11 and 12). Immediately after the diet was taken away on

Figure 11: Responding in pre- and post-diet testing.

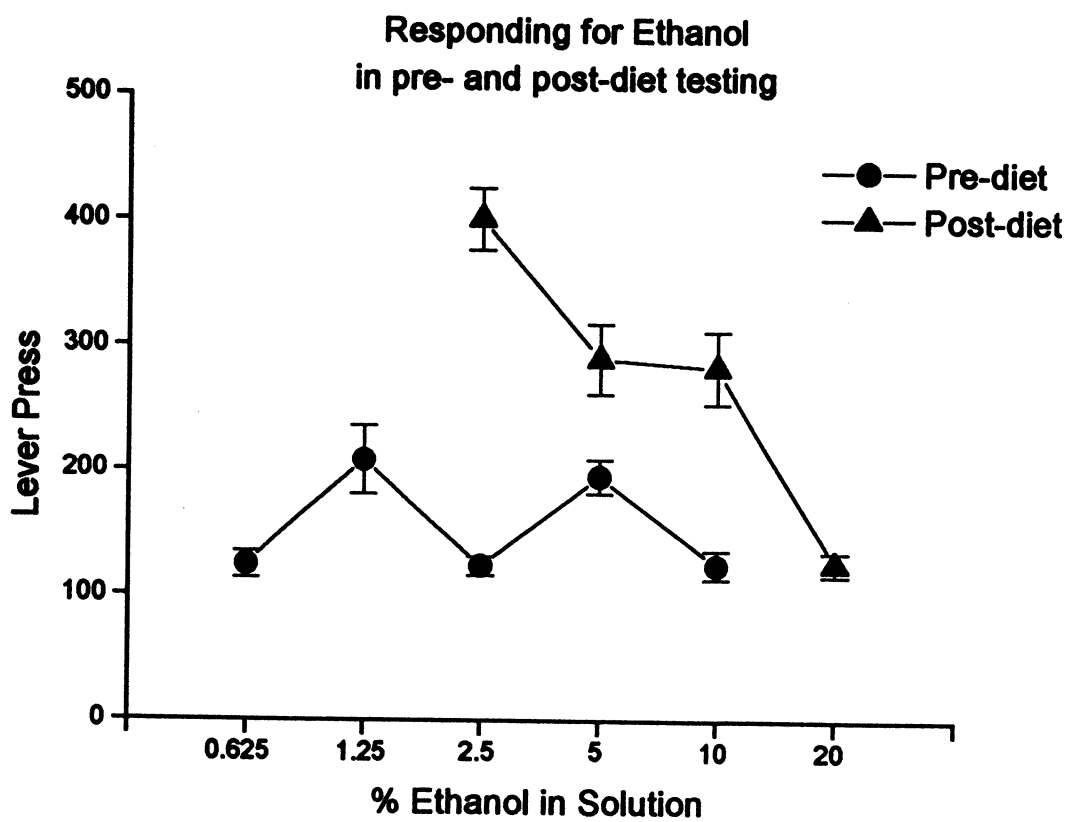


Figure 12: Ethanol intake in pre- and post-diet testing. Maximum daily intake was 10.56 g/kg. Repeated measures analysis of variance showed that both ethanol concentration and pre- vs. post-diet were statistically significant ($p < 0.001$).

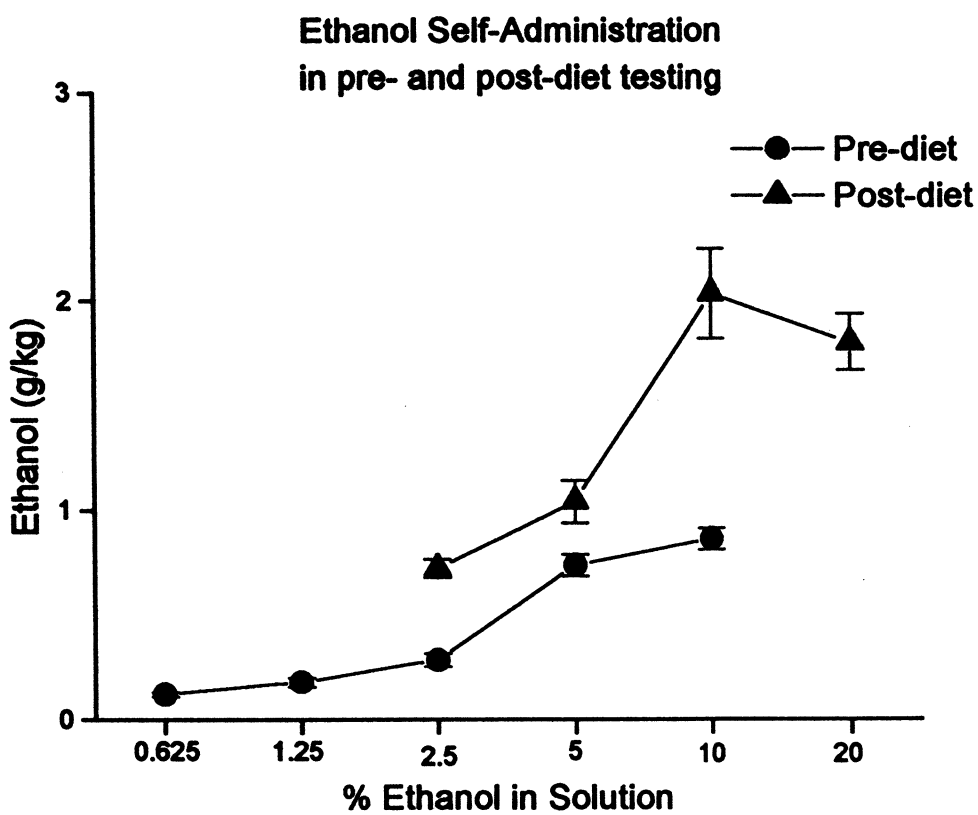
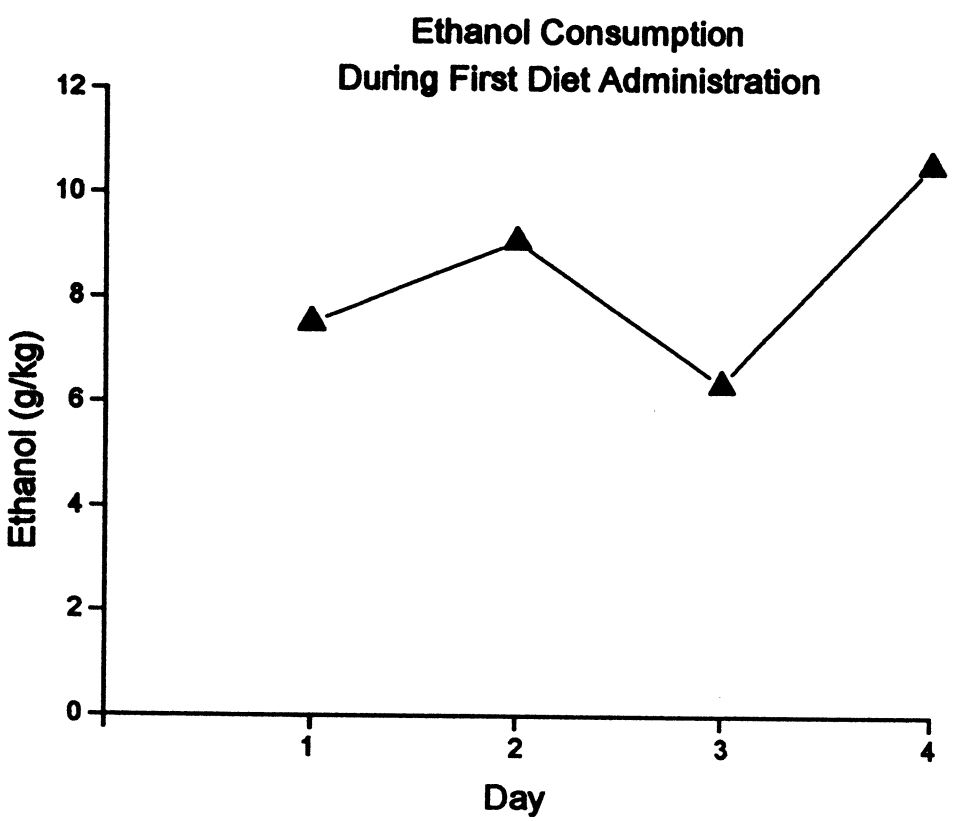


Figure 13: Ethanol intake during first liquid diet administration.



day 4, testing was attempted with 2.5% ethanol. Response levels were below 25 lever presses for all animals. Animals were fluid deprived with food pellets available for 24 hours, then retested. After the 30 minute test session, water was available in the home cage for 1 hour during the 2 days of testing. Mean lever pressing was 401 at 2.5% ethanol, 289 at 5% ethanol, 283 at 10% ethanol and 125 at 20% ethanol. Ethanol intake also rose after the liquid diet was administered to 0.72 g/kg at 2.5% ethanol, 1.04 g/kg at 5% ethanol, 2.03 at 10% ethanol and 1.80 at 20% ethanol. Repeated measures analysis of variance showed that both ethanol concentration and pre-diet vs post-diet ethanol intake were statistically significant ($p<0.001$).

During testing after ethanol liquid diet administration, lever pressing was recorded throughout the 30 minute session. Responding was divided into 5 minute time intervals. The greatest lever pressing occurred during the first 10 minutes of each session (Table 4). Several animals refrained from lever pressing for a few minutes in the middle of the session, then resumed pressing. A 2-way analysis of variance showed that both the ethanol concentration and time interval were statistically significant ($p<0.001$).

Second Diet Administration

The 8 rats which had been used for ethanol training and behavioral testing were again fed the liquid diet containing ethanol. In addition, 7 rats which had not been previously exposed to ethanol were fed a calorically

Table 4: Pattern of lever pressing for 5 minute intervals during post-diet testing.

Ethanol	Time	Individual Lever Presses								X	StD
2.5	5	43	42	52	59	39	44	43	37	45	6.7
2.5	10	24	33	53	35	29	26	25	33	32	8.2
2.5	15	31	18	23	40	19	1	13	16	20	10.2
2.5	20	5	1	0	4	13	0	10	12	6	5.0
2.5	25	0	0	0	0	0	0	0	5	1	1.6
2.5	30	0	0	0	0	9	2	7	2	3	3.2
10	5	18	29	37	39	37	36	39	25	33	4.7
10	10	29	17	16	0	32	18	12	22	18	8.4
10	15	24	0	1	22	12	0	25	10	12	9.1
10	20	43	4	10	0	8	0	9	10	11	4.2
10	25	13	13	12	2	26	12	12	11	13	6.1
10	30	0	0	4	6	13	12	15	13	8	4.9
20	5	13	24	22	18	23	24	9	24	20	4.8
20	10	7	5	4	3	8	6	13	10	7	3.1
20	15	0	4	11	1	2	0	4	2	3	3.1
20	20	14	3	0	0	1	1	7	1	3	2.2
20	25	0	6	0	1	8	2	2	8	3	2.9
20	30	0	0	2	2	7	2	1	2	2	1.9

balanced diet without ethanol. After 3 days, rats fed the nonethanol diet consumed all 100 mL of the diet allotment (Figure 14). Diet consumption varied for ethanol rats as shown also in Figure 14. Consequently, ethanol intake also varied (Figure 15). The highest ethanol consumption possible was 10.56 g/kg/day, (i.e., all 100 mL of diet consumed). Mean ethanol consumption for the 8 rats was 10.56 g/kg/day on days 8 and 14. Nonethanol rats comsumed all

Figure 14: Liquid diet consumed during second administration.

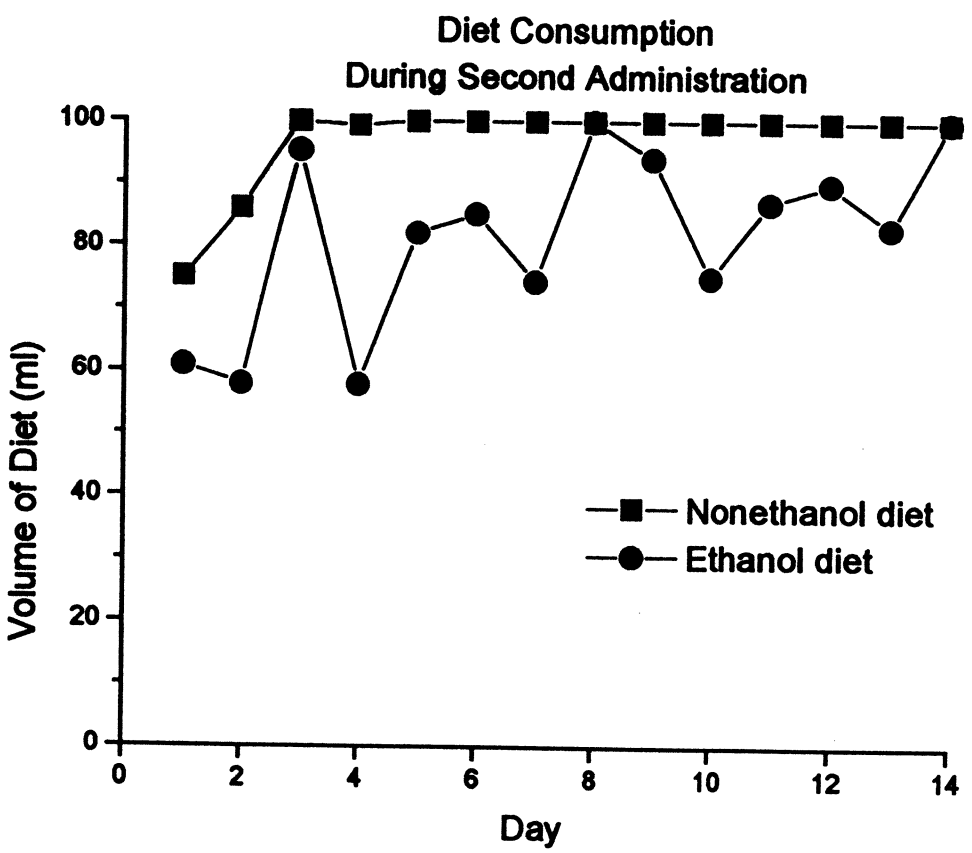
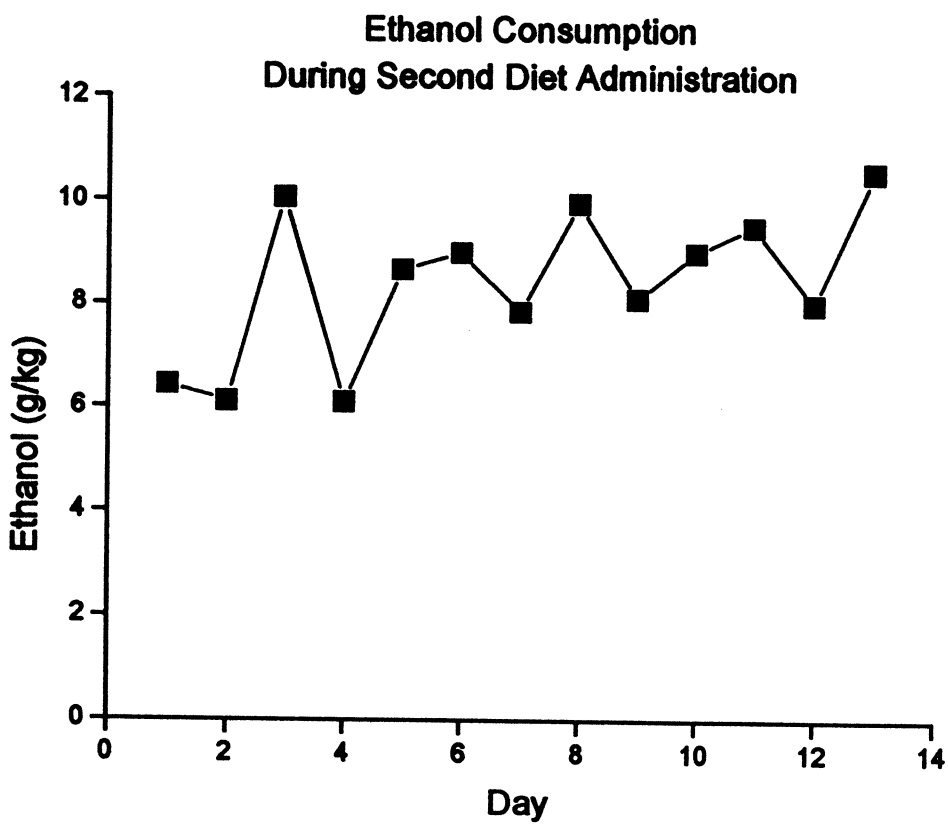


Figure 15: Ethanol intake during second liquid diet administration.



their daily allotment upon presentation. Ethanol rats consumed diet throughout the 24 hour period.

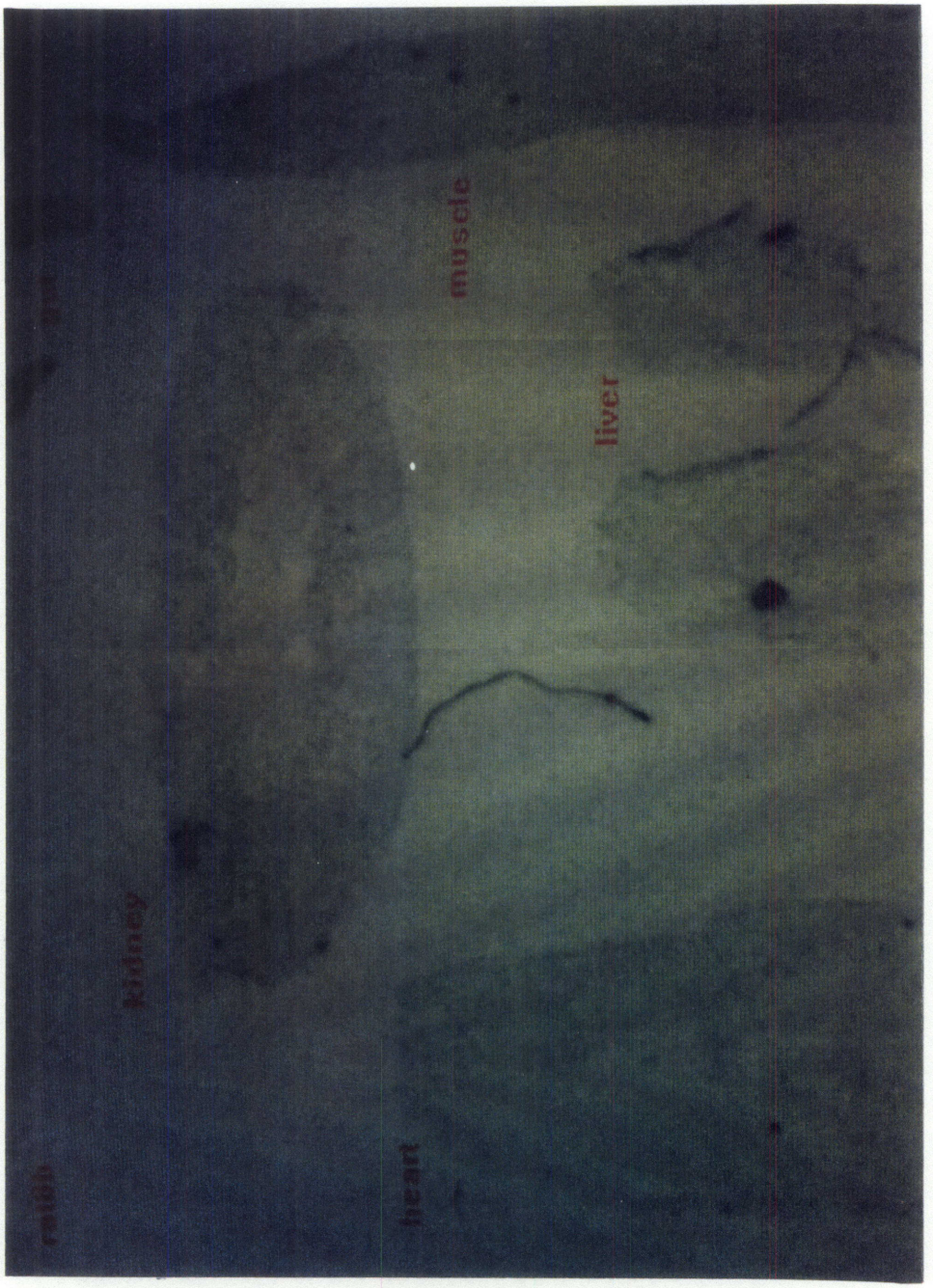
Ethanol rats had been handled extensively for several months prior to the second diet administration, whereas the nonethanol rats had not been handled as extensively. For a week prior to the diet administration, all rats were handled daily. Rats were also acclimated to the transport cart at this time. Handling was continued after the diet was administered. Through day 4, all rats passively accepted handling as they had prior to the second diet administration. Beginning on day 5, both ethanol and nonethanol rats showed signs of anxiety and aggression during handling (vocalizations, running, biting). Handling was discontinued. Daily observations of the animals' behavior occurred while feeding tubes were replaced in the cages.

Prior to tissue harvesting, animals were handled in the loading of the transport cart. Animals from both groups were resistant to handling and showed signs of anxiety: squirming, biting, and vocalizations.

In situ Hybridization

The pattern of radiolabeling was uniform throughout gastrointestinal, heart, skeletal muscle and liver tissue sections with both the constitutive and inducible probes (Figure 16). In kidney tissue, however, the constitutive probe showed a specific pattern of radiolabeling, with highest radiolabeling in the medulla (Figure 16). The inducible probe showed uniform labeling of kidney

Figure 16: Radiolabeling of tissue sections with the HSC70 probe. Gastrointestinal (gut), heart, muscle, liver and kidney sections are labeled. Note the distinctive pattern of labeling in the kidney, with highest radiolabeling in the medulla. The very dark artifacts were not included in samples analyzed.



tissue sections (Figure 17). Brain sections were divided into the following regions: hippocampal stellate cells, CA1, CA2, CA3, septum, cerebellar granule cells, cerebellar molecular layer, cortex, medial prefrontal cortex, dentate gyrus, diencephalon, striatum, thalamic nuclei, presubiculum and choroid plexus (Figures 10 and 18). The number of samples taken in a 10 x 13 pixel area and descriptive statistics for each tissue and region are shown in Table 6. Highest radiolabeling occurred in the hippocampal CA1-3, dentate gyrus, cerebellar granule cell layer and presubiculum.

Figure 19 shows median radiolabeling for each tissue and region under the four conditions of tissue from nonethanol rats labeled with the constitutive probe (NC), tissue from nonethanol rats labeled with the inducible probe (NI), tissue from ethanol rats labeled with the constitutive probe (EC) and tissue from ethanol rats labeled with the inducible probe (EI).

The constitutive probe showed radiolabeling in all tissue types of both nonethanol and ethanol rats, as expected. The inducible probe also showed radiolabeling in all tissues from both nonethanol and ethanol rats, a somewhat unexpected finding. In gastrointestinal, heart, skeletal muscle, liver and kidney tissues, median radiolabeling of mRNA for HSC70 and HSP70 was greater in ethanol rats than in nonethanol rats. In brain tissue, however, tissue from nonethanol rats showed greater median radiolabeling of HSC70 and HSP70 mRNA than ethanol rats in all regions except the cerebellar molecular layer. A 2-way analysis of variance over all tissues and regions showed that both diet

Figure 17: Radiolabeling of tissue sections with the HSP70 probe. Sections are labeled as for Figure 16. Kidney shows uniform radiolabeling with the inducible probe. Artifacts were not included in samples analyzed.

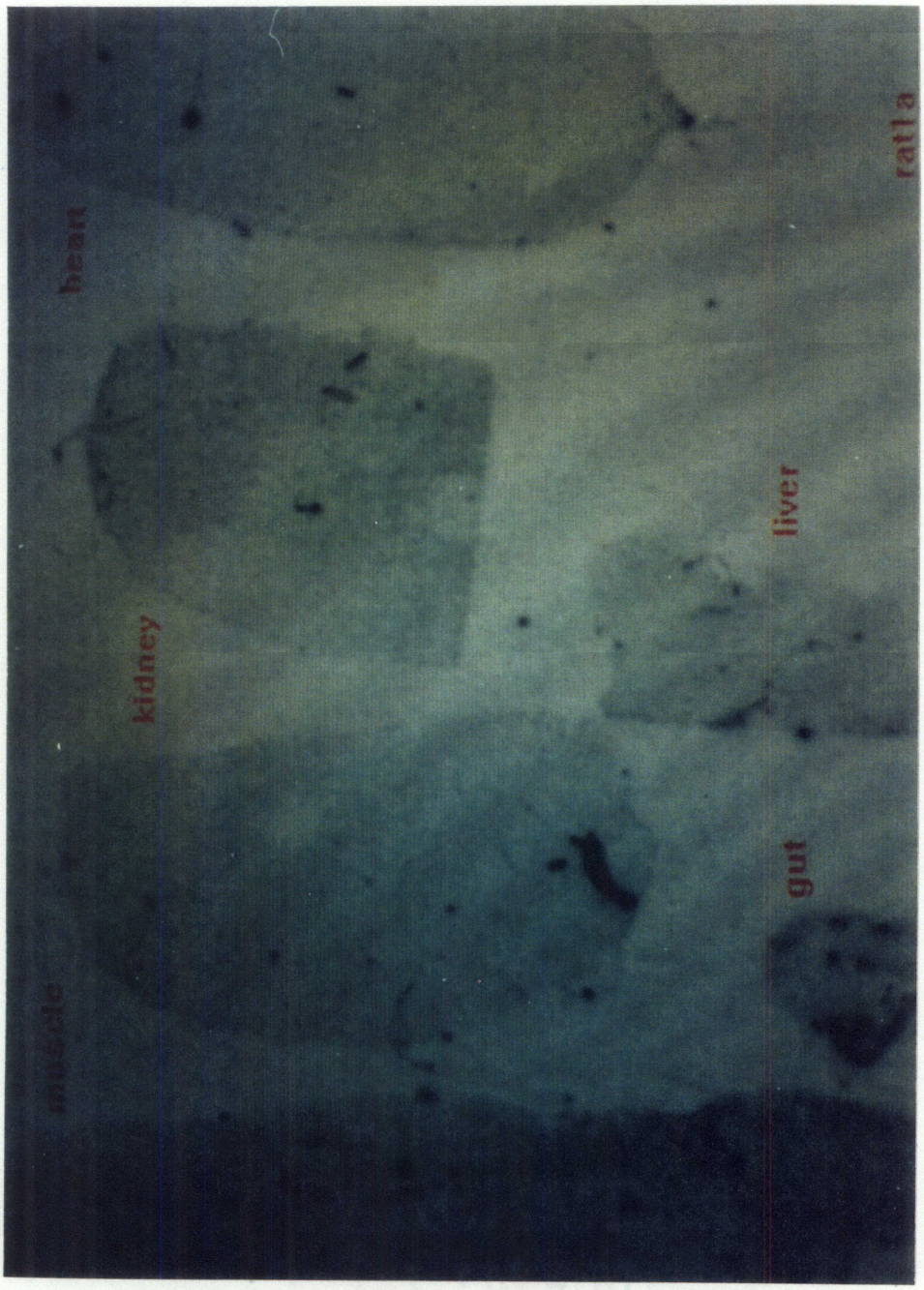


Figure 18: Radiolabeling of brain tissue section. The pattern of labeling was the same with the constitutive and inducible probes. Artifacts were not included in samples which were analyzed.

Table 5: Descriptive statistics for radiolabeling by tissue region and group, with the maximum, median, and minimum values. NC is tissue from nonethanol rats labeled with the constitutive HSC70 probe. NI is tissue from nonethanol rats labeled with the inducible HSP70 probe. EC is tissue from ethanol rats labeled with the constitutive probe. EI is tissue from ethanol rats labeled with the inducible probe. N is the number of samples analyzed from all rats meeting the group criterion.

Region	Group	Max	Median	Min	Num
Gastrointestinal	NC	31.88	19.73	7.50	70
Gastrointestinal	NI	38.01	22.83	14.69	70
Gastrointestinal	EC	42.26	22.48	9.35	80
Gastrointestinal	EI	36.11	26.61	10.11	79
Heart	NC	11.77	8.54	5.43	60
Heart	NI	20.08	10.75	7.42	60
Heart	EC	12.68	9.96	6.80	80
Heart	EI	22.25	11.22	7.57	80
Skeletal Muscle	NC	21.32	10.82	7.12	139
Skeletal Muscle	NI	27.98	10.41	6.31	140
Skeletal Muscle	EC	27.06	10.50	6.39	159
Skeletal Muscle	EI	28.52	12.50	6.74	160
Liver	NC	11.27	9.20	6.81	70
Liver	NI	33.95	12.24	5.26	70
Liver	EC	39.26	11.22	6.75	80
Liver	EI	41.61	15.23	5.85	80

Region	Group	Max	Median	Min	Num
Kidney Cortex	NC	14.98	9.94	7.23	60
Kidney Cortex	NI	54.58	12.21	8.36	69
Kidney Cortex	EC	13.96	10.28	6.41	79
Kidney Cortex	EI	32.51	14.54	8.68	79
Kidney Papilla	NC	12.55	9.63	6.95	31
Kidney Papilla	NI	29.14	19.4	14.5	14
Kidney Papilla	EC	22.40	9.92	6.52	41
Kidney Papilla	EI	45.82	28.72	12.09	16
Kidney Medulla	NC	35.99	16.19	8.19	58
Kidney Medulla	NI	29.01	23.45	20.84	5
Kidney Medulla	EC	20.99	15.15	8.48	66
Kidney Medulla	EI	25.79	18.61	14.11	22
Hippocampus Stellate	NC	36.80	20.35	5.28	136
Hippocampus Stellate	NI	45.25	14.74	5.64	135
Hippocampus Stellate	EC	53.66	17.75	5.37	146
Hippocampus Stellate	EI	59.93	21.4	5.37	191
Hippocampus CA1	NC	60.67	37.59	9.59	174
Hippocampus CA1	NI	83.23	41.22	6.14	161
Hippocampus CA1	EC	69.30	25.01	5.48	233
Hippocampus CA1	EI	63.34	26.43	5.51	312
Hippocampus CA2	NC	67.04	42.09	7.89	77
Hippocampus CA2	NI	76.70	35.57	13.05	69
Hippocampus CA2	EC	41.74	14.19	5.62	91
Hippocampus CA2	EI	74.34	29.38	5.65	95
Hippocampus CA3	NC	78.82	38.30	7.76	164
Hippocampus CA3	NI	87.30	34.32	7.65	126
Hippocampus CA3	EC	55.76	14.88	5.54	199
Hippocampus CA3	EI	63.76	24.12	5.68	183

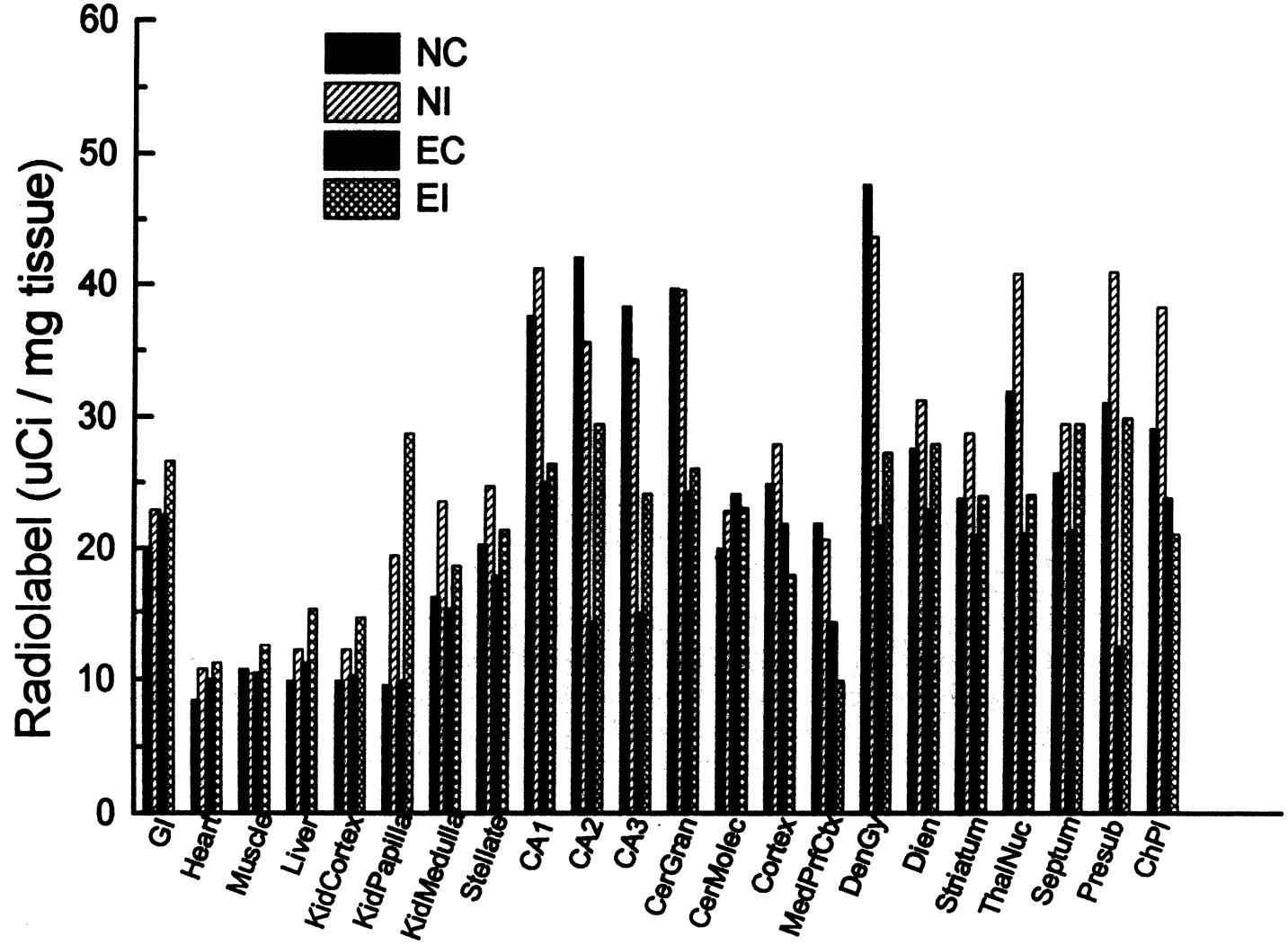
Region	Group	Max	Median	Min	Num
Cerebellar Granule Cells	NC	57.09	39.67	19.51	137
Cerebellar Granule Cells	NI	76.76	39.53	13.71	142
Cerebellar Granule Cells	EC	61.57	24.27	5.44	158
Cerebellar Granule Cells	EI	64.63	26.04	5.90	166
Cerebellar Molecular Layer	NC	33.55	20.03	6.00	136
Cerebellar Molecular Layer	NI	51.11	22.80	5.26	136
Cerebellar Molecular Layer	EC	52.28	24.09	5.62	174
Cerebellar Molecular Layer	EI	64.05	23.02	6.52	167
Cerebral Cortex	NC	44.78	24.91	7.93	395
Cerebral Cortex	NI	58.64	27.90	5.44	378
Cerebral Cortex	EC	61.77	21.76	5.28	231
Cerebral Cortex	EI	47.71	17.93	5.71	195
Medial Prefrontal Cortex	NC	30.63	21.93	5.56	111
Medial Prefrontal Cortex	NI	46.46	20.63	5.30	114
Medial Prefrontal Cortex	EC	28.25	14.22	5.43	20
Medial Prefrontal Cortex	EI	15.04	9.87	6.22	20
Dentate Gyrus	NC	81.59	47.50	23.15	186
Dentate Gyrus	NI	99.11	43.63	11.35	210
Dentate Gyrus	EC	65.09	21.66	5.28	209
Dentate Gyrus	EI	48.88	27.22	5.26	197
Diencephalon	NC	39.82	27.61	5.77	133
Diencephalon	NI	59.59	31.22	10.96	134
Diencephalon	EC	55.46	22.85	6.45	170
Diencephalon	EI	72.84	22.90	5.8	156
Striatum	NC	36.00	23.83	7.13	130
Striatum	NI	45.93	28.72	7.83	129
Striatum	EC	52.48	20.88	5.49	156
Striatum	EI	79.46	28.37	6.03	176

Region	Group	Max	Median	Min	Num
Thalamic Nuclei	NC	50.15	31.94	14.20	81
Thalamic Nuclei	NI	69.15	40.80	15.14	56
Thalamic Nuclei	EC	40.16	21.13	5.62	50
Thalamic Nuclei	EI	54.43	23.99	11.82	20
Septum	NC	43.39	25.73	7.49	80
Septum	NI	53.24	29.40	8.48	60
Septum	EC	45.2	21.34	5.44	81
Septum	EI	53.14	29.44	6.81	115
Presubiculum	NC	47.43	31.10	9.88	39
Presubiculum	NI	76.43	42.86	15.11	71
Presubiculum	EC	49.99	12.40	5.69	105
Presubiculum	EI	76.13	29.90	6.73	110
Choroid Plexus	NC	59.73	29.05	13.55	65
Choroid Plexus	NI	73.71	38.24	14.13	51
Choroid Plexus	EC	54.50	23.77	5.65	107
Choroid Plexus	EI	89.12	20.97	5.45	141

and probe type were statistically significant ($p<0.0001$), but the interaction of diet and probe type was not ($p=0.38$). Mean radiolabeling for all nonethanol tissues labeled with the constitutive probe was 25.95 µCi/mg tissue with standard deviation of 14.182. For all nonethanol tissues radiolabeled with the inducible probe, mean and standard deviation were 28.80 +/- 16.123 µCi/mg tissue. In tissues from ethanol rats, the constitutive probe showed mRNA radiolabeling mean and standard deviation of 20.64 +/- 12.421 µCi/mg tissue and the inducible probe, 23.81 +/- 13.006 µCi/mg tissue.

Figure 19: Median radiolabeling of tissue regions in each group. NC is tissue from nonethanol rats labeled with the constitutive HSC70 probe. NI is tissue from nonethanol rats labeled with the inducible HSP70 probe. EC is tissue from ethanol rats labeled with the constitutive probe. EI is tissue from ethanol rats labeled with the inducible probe. Tissue regions are listed in the order they appear in Table 5.

Median Radiolabel for each Tissue by Group



Heart, muscle, liver and kidney cortex tissue showed lowest median radiolabeling of mRNA for HSC70 and HSP70 in nonethanol and ethanol rats, with tissues labeled by the inducible HSP70 probe showed greatest radiolabeling in ethanol rats. Kidney papilla showed similar levels of constitutive probe labeling in tissue from both nonethanol and ethanol rats. The inducible probe showed much greater labeling in kidney papilla than heart, muscle, liver or kidney cortex tissue, however, with median inducible labeling of ethanol tissues the highest. In kidney medulla, the median constitutive mRNA labeling was double the median constitutive labeling in kidney cortex or papilla (Figure 19). Again, however, the inducible HSP70 probe showed greater labeling than constitutive, highest in nonethanol rats. Median labeling of gastrointestinal tissue was double that of heart, muscle or liver for the 4 groups, with EI showing the greatest median radiolabeling.

In general, brain regions showed greater median mRNA radiolabeling than heart, muscle, liver or kidney tissue. Also, brain tissue sections from nonethanol rats showed greater radiolabeling than ethanol rat brain tissue, except in the cerebellar molecular layer and septum.

Median labeling of the constitutive probe in tissue from nonethanol rats was greater than NI, EC, or EI in hippocampus CA2 and CA3, cerebellar granule cells, medial prefrontal cortex, and dentate gyrus. Inducible mRNA labeling was greatest in hippocampal stellate cells and CA1, cortex,

diencephalon, striatum, thalamic nuclei, presubiculum and choroid plexus. Constitutive probe labeling was greatest in ethanol rats only in the cerebellar molecular layer, and inducible labeling of ethanol tissues was equal to inducible nonethanol labeling in septum.

Kruskal - Wallis nonparametric analysis of variance was performed to compare the 4 groups (NC, NI, EC, and EI) in each tissue region. In all regions, the 4 groups were statistically significantly different ($p<0.05$). To determine the relationships between the groups, ranked data was used for Tukey's multiple range test (Table 6).

Gastrointestinal, heart, liver, CA2, cerebellar molecular layer, dentate gyrus, and choroid plexus showed statistically significantly different labeling of NI, EC and EI from NC ($\alpha=0.05$). Kidney cortex and papilla, hippocampal stellate cells, and striatum tissue showed NI and EI as statistically significantly different from NC ($\alpha=0.05$), but EC was not. In kidney medulla and diencephalon tissues, only NI was statistically significantly different from NC ($\alpha=0.05$). In muscle and medial prefrontal cortex, only tissue from ethanol rats labeled with the inducible probe was statistically significantly different from NC ($\alpha=0.05$).

Radiolabeling of mRNA in each group was statistically separate from each other in Tukey's test on ranked data in tissue from heart, hippocampal CA2 and dentate gyrus. In gastrointestinal tissue, ranked radiolabeling of NI,

Table 6: Separation of median radiolabeling in each tissue region. Underlining indicates groups that were not statistically separate ($\alpha = 0.05$).

Region	Separation by Tukey's test
Gatrointestinal	EI NI EC > NC
Heart	EI NI > NC > EC
Muscle	EI > NI NC > NC EC
Liver	EI > EC NI > NC
Kidney Cortex	EI NI > EC NC
Kidney Papilla	EI > NI > EC NC
Kidney Medulla	NI EI > EI NC EC
Hippocampal Stellate Cells	EI NI EC > NI EC NC
CA1	NC NI > EI EC
CA2	NC > NI > EI > EC
CA3	NC CI > EI > EC
Cerebellar Granule Cells	NC NI > EI EC
Cerebellar Molecular Layer	EI EC NI > NC
Cortex	NI NC > EC EI
Medial Prefrontal Cortex	NI NC EC > EC EI
Dentate Gyrus	NC > NI > EI > EC
Diencephalon	NI > NC EI EC
Striatum	EI NI > NC EC
Thalamic Nuclei	NI NC > EI EC
Septum	NI > EI NC EC
Presubiculum	NI > EI NC > EC
Choroid Plexus	NI > NC > EC EI

EC, and EI were separate from NC, but not from each other. In hippocampal CA1 and CA3, cerebellar granule cells, cortex, and thalamic nuclei tissue, NC was not separate from NI, nor was EC separate from EI, although the nonethanol groups were statistically separate from the ethanol groups.

To compare mRNA labeling with either the constitutive or the inducible probe between nonethanol and ethanol tissue in each region, change from nonethanol to ethanol was computed as a percentage of the nonethanol labeling (Figures 20 and 21). Wilcoxon rank sums nonparametric test was also conducted to determine if mRNA labeling with either the constitutive or inducible probe was statistically significantly different in tissues from nonethanol or ethanol rats (Figures 20 and 21).

In gastrointestinal, heart, and liver tissue, mRNA labeling with the constitutive probe increased in ethanol rats as compared with nonethanol rats and the difference in radiolabeling was statistically significantly different ($p<0.05$). In all brain regions except the cerebellar molecular layer, median mRNA labeling with the constitutive probe was lower in ethanol rats than nonethanol rats. The greatest change occurred in brain tissue, particularly the hippocampal regions, dentate gyrus, presubiculum and cerebellar granule cells. In these regions, also, mRNA labeling with the constitutive probe was highly statistically significantly different ($p<0.001$) in tissue from ethanol rats and nonethanol rats. Changes in mRNA labeling with the inducible probe from nonethanol tissues to ethanol tissues were much more pronounced in

Figure 20: Percent change in median mRNA labeling with the constitutive (HSC70) probe in tissue from nonethanol and ethanol rats ((EC-NC)/NC*100). Wilcoxon ranked sums nonparametric analysis was also performed. * indicates statistically significant difference in mRNA labeling between tissues from nonethanol and ethanol rats ($p<0.05$). ** indicates highly stastically significant difference ($p<0.001$) in mRNA labeling in tissues from nonethanol and ethanol rats.

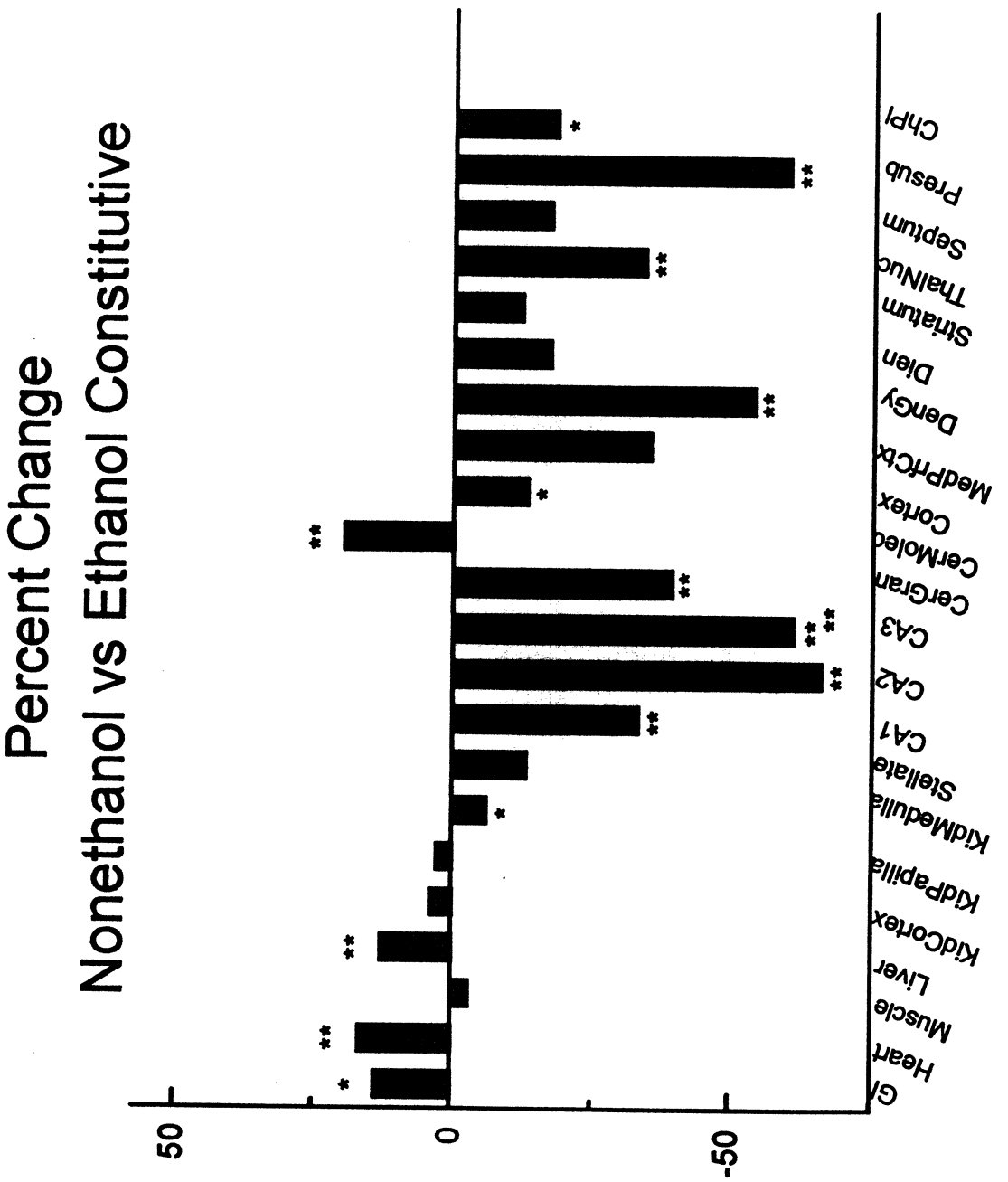
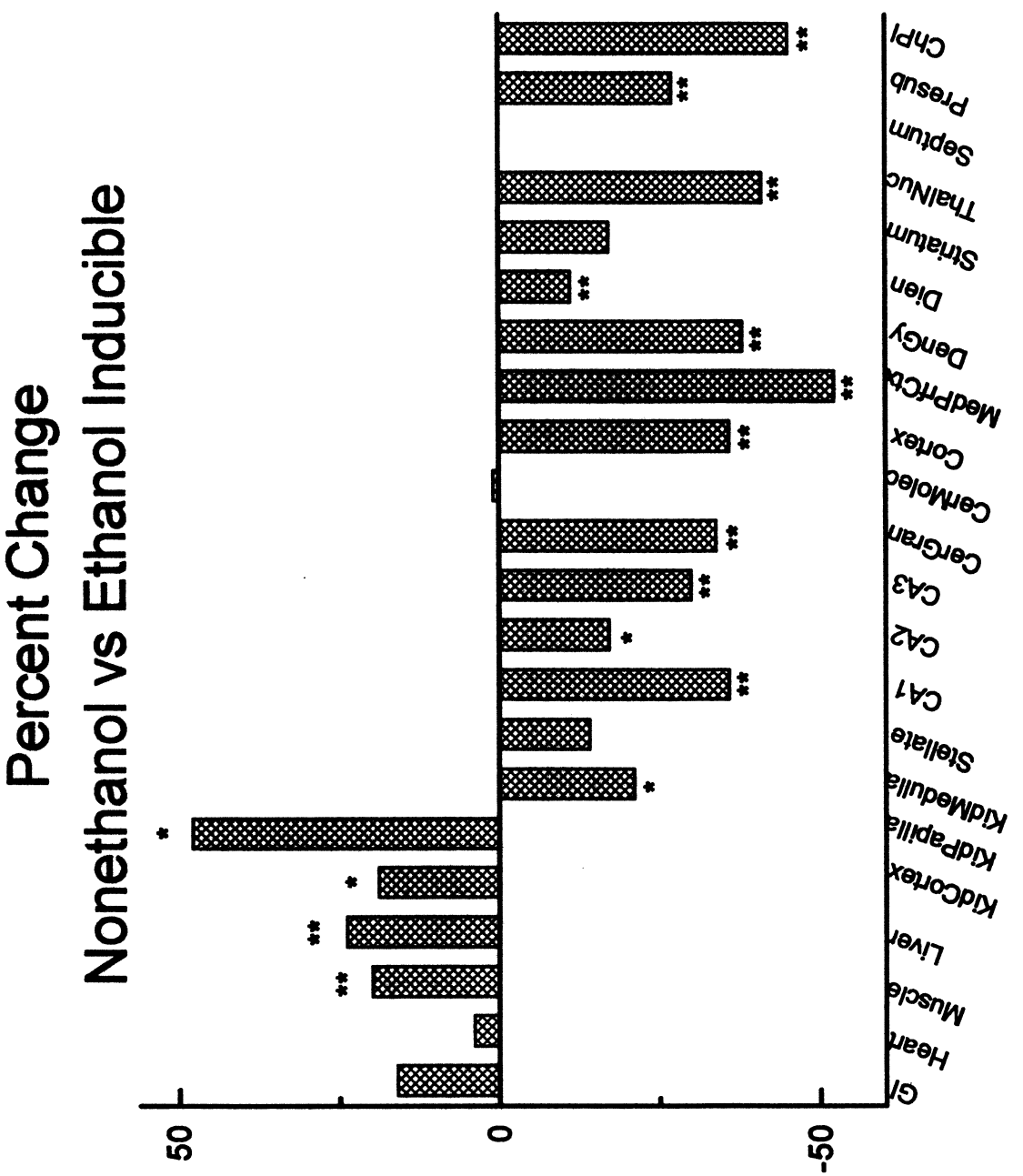

Percent Change
Nonethanol vs Ethanol Constitutive
50
0
-50
Gl
Heart
Muscle
Liver
KidCortex
KidPapilla
KidMedulla
Stellate
CA1
CA2
CA3
CerGran
CerMolec
Cortex
MedPrfCtx
DenGy
Dien
Striatum
ThalNuc
Septum
Presub
ChPl

Figure 21: Percent change in median mRNA labeling with the inducible (HSP70) probe in tissue from nonethanol and ethanol rats ((EI-NI)/NI*100). Wilcoxon ranked sums nonparametric analysis was also performed. * indicates statistically significant difference in mRNA labeling between tissues from nonethanol and ethanol rats ($p<0.05$). ** indicates highly stastically significant difference ($p<0.001$) in mRNA labeling in tissues from nonethanol and ethanol rats.


Percent Change
Nonethanol vs Ethanol Inducible
50
0
-50
Gl
Heart
Muscle
Liver
KidCortex
KidPapilla
KidMedulla
Stellate
CA1
CA2
CA3
CerGran
CerMolec
Cortex
MedPrfCtx
DenGy
Dien
Striatum
ThalNuc
Septum
Presub
ChPl

gastrointestinal, muscle, liver and kidney tissue. Statistical significance was obtained in muscle, liver, kidney cortex and papilla, with an increase in labeling in ethanol rats over labeling in nonethanol rats. In kidney medulla, as well as most of the brain regions, median mRNA labeling with the inducible probe was lower in tissues from ethanol rats than nonethanol rats. The greatest change was in medial prefrontal cortex, choroid plexus, thalamic nuclei, dentate gyrus, cortex, cerebellar granule cells, and hippocampal CA1 and CA3, with highly statistically significant differences between ethanol and nonethanol tissues ($p<0.001$).

CHAPTER 4

DISCUSSION

In examining the results, the 5 major observations were (i) ethanol intake increased after the first diet administration. (ii) The pattern of ethanol intake seems to indicate that the animals were limiting their ethanol intake in such a way to titrate blood ethanol levels to a preferred level. (iii) *In situ* hybridization showed greater radiolabeling with both the inducible and constitutive probes in heart, liver, gastrointestine, skeletal muscle and kidney tissues from ethanol rats over those same tissues from nonethanol rats. (iv) In brain tissue, nonethanol rat tissue showed greater radiolabeling than brain tissue from ethanol rats. (v) The direction of change from ethanol to nonethanol radiolabeling (i.e., an increase or decrease in radioactivity) was the same with the constitutive or inducible probe.

Ethanol Training and Testing

Using sucrose fading in ethanol solutions worked well to initiate responding for ethanol. However, Fischer 344 rats showed very little responding for ethanol alone after they were acclimated to taking ethanol with sucrose. The stimulus to self-administer ethanol does not seem strong enough to overcome the negative stimulus of the ethanol without sucrose in this specific

strain of rats, which supports the conclusions of George (1987) and Suzuki et al., (1988).

Alternatively, animals can be acclimated to a solution of sucrose and ethanol; saccharin can gradually replace sucrose in the solutions. Using saccharin would eliminate the caloric contribution of sucrose in the stimulus to the self-administration of ethanol. Another option would be to acclimate animals to ethanol via a home cage bottle test in which animals are given the choice of ethanol or water in the home cage; after which those rats preferring ethanol could then be trained to self-administer ethanol via lever pressing. If an additional sweetener is required to induce the animals to administer ethanol, saccharin can be used, but was not used in this paradigm.

Self-administration of ethanol increased after 4 days of ethanol liquid diet administration, indicating that there was some effect on ethanol intake following the chronic exposure. Rats fed ethanol liquid diet consumed the aliquot of diet throughout the 24 hour period, which maintained the constant presence of ethanol in the body. Rats self-administered approximately the same concentration of ethanol in daily training. Over time, the daily level of ethanol intake rose gradually.

It has been suggested that self-administering animals titrate their blood ethanol levels (Samson and Grant, 1990). This is consistent with the decreased lever pressing at higher ethanol concentrations because a smaller volume of fluid delivers the ethanol concentration necessary to reach the animal's

preferred blood ethanol level. The time course of ethanol consumption during a 30 minute test session also supports the titration of blood ethanol levels, in that consumption occurs in time bins. Highest responding for ethanol occurred during the first 10 minutes of the test session, perhaps until blood ethanol levels reached a certain preferred level for each individual. Responding could fall off while blood ethanol stays at the preferred level, with higher levels of responding resuming when blood ethanol levels dropped below the preferred level.

The consumption of the liquid diet throughout the day also supports the titration of blood ethanol levels. Animals could consume the diet until a negative feedback, possibly the intoxicating effects of ethanol, turned off the stimulus to continue consuming the diet. As sustained behavioral effect of ethanol increased, such that a larger dose of ethanol was needed to produce the same level of intoxication, so could the consumption of ethanol liquid diet. High levels of ethanol consumed could have resulted in a negative reaction to the increased consumption; with the negative reinforcement, ethanol consumption would decline. As the behavioral effect developed again, consumption could increase, as is the case in the consumption of liquid diet during the second administration.

Majchrowitz (1975) concluded that elevated levels of blood ethanol for 4 days produces tolerance to the effects of ethanol. Tolerance could be achieved through many mechanisms: changes in receptor sensitivity or density and changes in neurotransmitters such as dopamine or adrenal corticotropic

hormones (Watson, 1990). Other mechanisms remain viable to explain the effect.

Traditionally, in interpreting dose effect curves, a shift to the right in the dose effect curve indicates the development of tolerance. A shift to the left in the dose effect curve indicates sensitization. In this study, the dose effect curve was shifted upwards, indicating that a larger amount of ethanol was needed to obtain the desired behavioral effect. It is difficult at this time to define the behavioral change in ethanol consumption as either behavioral tolerance or behavioral sensitization. However, after long term exposure to the ethanol diet, subjects showed an increase in ethanol intake.

In situ hybridization of mRNA for HSC70 and HSP70

Highest labeling of mRNA for HSC70 and HSP70 occurred in brain regions (e.g., hippocampal CA1-3, dentate gyrus, thalamic nuclei, presubiculum, choroid plexus and cerebellar granule cells) which is consistent with other reports (Blake et al., 1990; Kawagoe et al., 1992; Manzerra and Brown, 1992; Nowak, 1990). In general, brain tissue showed higher labeling of HSP mRNA than the gastrointestinal, heart, muscle, liver or kidney tissue. This is consistent with the results of Blake et al. (1990) that HSP levels in brain were higher than in liver.

The presence of the inducible mRNA labeled by the inducible HSP70 probe in tissue from nonethanol rats could be the result of handling the animals.

In adrenal gland and aorta tissue, the minimal stress associated with handling and injecting animals was sufficient to induce HSP70 in animals that had been previously acclimated to these procedures (Blake et al., 1994). By day 5 of the second diet administration, both nonethanol and ethanol rats were extremely resistant to handling. This anxiety could have been the result of the behavioral changes in the ethanol rats from the effects of ethanol diet causing intoxication and the potential of withdrawal. Although the animals were housed in separate cages, the cages were in close proximity and the animals are extremely sensitive to behaviors of animals in nearby cages. By day 5, rats consuming the ethanol diet were beginning to develop behavioral changes, possibly tolerance, to the effects of the constantly raised blood ethanol levels, which may have precipitated behavioral changes which would, in turn, also affect the nonethanol rats. In rats consuming ethanol, a period of hyperactivity is observed which would have affected both the ethanol and nonethanol rats (Samson and Grant, 1990).

Ethanol causes changes in receptor sensitivity and neurotransmitter levels (Watson, 1990). The HSPs could also be induced due to the direct actions of ethanol on the tissues, i.e., fluidizing the cell membrane. Changes in neurotransmitters may induce HSPs (Blake et al., 1994), particularly in areas influenced by the hypothalamic-pituitary-adrenal axis. If the effects of ethanol have induced HSPs, subsequent stresses would show less induction of HSPs. The HSPs already present would stabilize cellular components from subsequent

insult, leading to the development of stress tolerance. Also, HSP70 is a long lasting cellular protein. Following exposure to stress, HSPs may be present for as long as 10 days. Further, mRNA for HSC70 and HSP70 has a longer half-life after stress (Craig, 1985).

One explanation for the induced labeling of the nonethanol tissues is the anxiety produced by handling the animals prior to tissue harvesting. The HSP induction is very rapid, within 1 minute in cultured cells (Craig, 1985). Ethanol can exhibit an anxiolytic affect (Watson, 1990), which would decrease the release of anxiogenic neurotransmitters and subsequently decrease the stimulus for HSP induction. This might also account for the increase in labeling of ethanol tissues over nonethanol tissues in the peripheral organs, but not in the brain. Gastrointestinal, heart, muscle, liver and kidney tissue may not be affected in the same way as the brain by central nervous system stimulation.

While animals are receiving liquid diet, no other source of fluid is available. Dehydration may, therefore, also be a factor in the levels of HSC70 and induction of HSP70 in nonethanol animals.

The hypothesis being tested in this experiment was that levels of constitutive and inducible 70 kD stress protein mRNA would be different in ethanol and nonethanol rats *in vivo*. Miles et al., (1990) would predict an increase in HSC70 mRNA labeling, while Walsh and Crabb (1989) would predict an increase in HSP70 mRNA after the prolonged exposure to physiological levels of ethanol.

Both the inducible HSP70 and the constitutive HSC70 levels can increase after stress. The promoter region for HSC70 contains the heat shock element which would be stimulated to initiate transcription following stress (Sorger and Pelham, 1987). Miller et al., (1991) suggest that elevation in the constitutive HSC70 may be sufficient to protect cells from lower levels of stress. This may also explain the findings of Miles et al., (1990) that ethanol increases levels of HSC70 rather than the more commonly accepted view that ethanol increases levels of HSP70. With high levels of ethanol (>5% in culture media), induction of HSP70 would occur due to the severity of the physiological insult; while lower levels of ethanol would increase HSC70 to protect cellular components from the milder stress.

Changes that were statistically significantly different in tissue from ethanol rats compared to nonethanol rats in the constitutive and inducible probe radiolabeling within the same tissue region occurred in the same direction, as expected since the proteins perform the same functions: protecting cellular proteins from denaturation or abnormal aggregation.

Gastrointestinal tissues showed greatest median radiolabeling with the inducible probe in tissue from ethanol rats. In personal observation, Dr. Cleatis Wallis (University of North Texas Health Science Center at Fort Worth, personal communication) noted that the ethanol liquid diet caused a color change, a yellowing, in the gastrointestinal tract of ethanol rats. Rats receiving the nonethanol liquid diet showed no observable difference in gastrointestinal

tissue. Gastrointestinal tissue is constantly barraged with physiological insults: changes in ionic concentrations, pH, etc. Levels of HSPs in gastrointestinal tissue may be higher than in heart or muscle to protect cellular proteins from these insults. Ethanol is absorbed by gastrointestinal tissue which is in immediate contact with the highest levels of ethanol. The HSPs would be recruited to protect the cell from this stress (Nakamura et al., 1991), hence the increase in constitutive labeling in tissue from ethanol rats over nonethanol rats. Median inducible labeling was higher in tissues from ethanol rats than nonethanol rats, but this difference was not statistically significantly different. This would suggest that the liquid diet without ethanol increased HSPs, possibly due to dehydration.

The liver is primarily responsible for the detoxification of ethanol. Yet, liver did not show HSP levels as high as that of gastrointestinal tissue. Biochemical pathways in the liver which detoxify ethanol may be sufficient to protect the cells from damage without the higher levels of HSPs required by gastrointestinal tissue. Both the inducible and constitutive probes showed statistically significant labeling of mRNA in ethanol and nonethanol tissue, with median labeling in ethanol tissue higher.

In kidney cortex and papilla, highest median labeling occurred with the inducible probe in tissue from ethanol rats. Inducible probe labeling was statistically significantly different in ethanol and nonethanol tissues, with ethanol tissues showing higher median mRNA radiolabeling. The median constitutive

probe labeling was slightly higher in ethanol tissues than nonethanol tissues, but the difference was not statistically significant. The fact that the constitutive probe showed little change in labeling may indicate that the nonethanol tissues were expressing high levels of constitutive HSC70 due to the effects of the diet alone. If the animals were dehydrated, kidney could be especially sensitive to salt imbalances. The extremely high increase in inducible mRNA labeling in ethanol tissues over nonethanol tissues in the kidney papilla could be a direct result of the ethanol diet. The papilla contains membranes for the reabsorption of nutrients and salts back into blood. Ethanol in the blood would be likely to stress these cells in close contact with the ethanol.

In the kidney medulla, labeling of the constitutive and inducible probes was statistically significantly different in ethanol and nonethanol tissues. However, unlike kidney cortex and papilla, ethanol tissues showed a decrease in median labeling in ethanol tissues from nonethanol tissues, as did all brain regions except the cerebellar molecular layer. This may indicate functional differences in fluid biochemistry, possibly from dehydration, or a difference in central nervous system stimulation.

In muscle, the constitutive probe showed little change in labeling tissue from ethanol and nonethanol rats and this difference was not significant. Like the kidney cortex and papilla, this small change in constitutive labeling may be due to an already high expression of HSC70 in response to the diet itself. The

highly significant change (increase) in inducible probe labeling, however, could be attributable to ethanol.

Brain tissue in the cerebral cortex, medial prefrontal cortex and choroid plexus also showed little change in the constitutive labeling of nonethanol and ethanol tissues, with dramatic changes in labeling by the inducible probe. In these brain regions, ethanol tissues showed lower median labeling of mRNA than nonethanol tissues. If stress proteins had been induced by ethanol either by direct action on the tissue or by changes in neurotransmitters, stress tolerance would occur. Consequently, HSPs would already be present to stabilize cellular proteins against further stress. Less induction of HSPs would occur and less mRNA would be transcribed, resulting in a lower radioactive signal. Alternatively, if nonethanol rats had elevated levels of HSC70 or HSP70 due to anxiety, the anxiolytic effects of ethanol could result in less stimulation for induction of HSPs.

In heart tissue, constitutive labeling of ethanol and nonethanol tissue was highly significantly different ($p < 0.001$), with ethanol tissue showing higher median labeling. Inducible labeling was not significantly different in ethanol and nonethanol tissues. In rats receiving nonethanol diet, there was likely little stimulation for the induction of HSPs by the diet alone. Thus, the change in constitutive levels is likely due to the ethanol, which would indicate that ethanol increases expression of HSC70 in heart tissue. Cytoprotection of heart tissue against ischemic damage is an active area of research (Yellon and Latchman,

1992; Welch, 1993) in seeking a method to minimize damage from surgery or to increase the viability of isolated organs for transplantation. Information on HSP changes in these tissues may be useful in developing a therapeutic method to increase cytoprotection.

In hippocampal CA2 and CA3, dentate gyrus and presubiculum tissue, labeling with the constitutive and inducible probes were significantly different in tissues from ethanol and nonethanol rats. Nonethanol rat tissue showed higher median labeling with both probes than ethanol rat tissue. The magnitude of the change was greater in constitutive mRNA labeling than inducible mRNA labeling. These regions show the greatest labeling of HSP mRNA in controls or with many inducers including heat shock (Blake et al., 1990; Kawagoe et al., 1992; Manzerra and Brown, 1992; Nowak, 1990). Higher labeling in nonethanol tissue could be attributable to the anxiolytic neurochemical effects of ethanol.

Heat shock proteins are potent biomarkers of stress in cellular systems. Changes in stress proteins can help to determine mechanisms by which drug actions occur (Blake et al., 1994), or which cells are especially vulnerable to the effects of a drug. Higher levels of HSPs in brain regions may indicate that this organ is particularly vulnerable to physiological stresses, particularly ethanol insult. Lower levels of HSPs in the peripheral organs (i.e., heart, muscle, liver, etc.) may indicate that those tissues are less vulnerable to the effects of ethanol, possibly due to biochemical pathways which are recruited help to alleviate the stress insult. The decreased median labeling in tissues from

ethanol rats as compared to nonethanol rats could indicate that the brain is more vulnerable to the effects of ethanol because the presence of ethanol would activate the stress response. Stress tolerance could then protect the cells from further insult. Consequently, less mRNA would be transcribed because the levels of HSPs already exist in sufficient quantities to alleviate the effects of any subsequent stress insult.

As biomarkers, induction of HSPs can show tissue and organ injury following medical procedures, stroke or heart attack. In addition, stress proteins can be used to mark changes in an organism's environment (Welch, 1993). Stress proteins could be used to monitor the dangers of environmental pollutants with transgenic stress reporter microorganisms, which change color when subjected to stress that induces HSPs.

Environmental biologists have taken the role of stress proteins as "reporter genes" one step further. Monitoring stress protein levels in bioindicator species could provide information on the state of environmental systems (Sanders and Martin, 1993). Animals high in the food chain, such as marine mammals in oceanic ecosystems, make natural candidates for monitoring stress protein levels. Because the stress proteins and their genetic codes are so highly conserved throughout evolution, antibodies to the HSPs or probes developed for their mRNAs could be easily used as an assay tool.

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